Mechanisms underlying Retinoic acid-Induced Chemoattraction in Molluscan Neurons

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Abstract

Retinoic acid, a derivative of vitamin A, is known to play diverse roles in development and regeneration. Previous research in the mollusc *Lymnaea stagnalis* has shown that a gradient of *all-trans* retinoic acid attracts the growth cones of cultured neurons. The present study investigates the sub-cellular mechanisms within the growth cones of *Lymnaea* pedal A neurons which mediate the attractive response to a gradient of *all-trans* retinoic acid. In this study, the mechanism of growth cone turning is shown to be local, as neurites mechanically isolated from their cell body retain the capacity to turn towards an exogenous gradient of *all-trans* retinoic acid. The turning response is dependent on the initiation of protein synthesis and calcium influx, but does not appear to involve signaling through protein kinase C (PKC). The retinoid X receptor (RXR), which classically functions as a transcription factor, was also shown to be involved in the turning response, functioning locally through a non-genomic pathway. These data show, for the first time in any species, that *all-trans* retinoic acid's chemotropic action involves a local mechanism involving non-genomic signaling through the RXR. As retinoic acid is known to play a role in regeneration, understanding the mechanisms underlying retinoic acid signaling may lead to further advances in regenerative neuroscience.

Acknowledgements

John Donne famously wrote, "no man is an island entire of itself," thereby conveying the deep truth that we can never be at our best when isolated from others. This has certainly been the case during the course of my master's degree. I am grateful to have studied under two superb supervisors, Drs. Gaynor Spencer and Robert Carlone. Both have modeled both scientific and personal integrity, setting an example for me as I move forward in my own career.

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I would also like to thanks the members of my thesis committee, Dr. Joffre Mercier and Dr. Glenn Tattersall, for their helpful suggestions and insights during the course of my master's degree program. I also appreciate the time and effort of the faculty who taught me in my graduate courses (Drs. Spencer, Carlone, Mercier, Richards, and Haj-Amed). I also thankfully acknowledge the support I received from NSERC in the form of a master's scholarship. Finally, my laboratory colleagues, Chris Carter, Nick Vesprini and Cailin Rothwell have been a source of ongoing support and assistance.

While I will miss working with all whom I have had the pleasure of interacting over the past 2 years, I am confident that the skills I have acquired during this period will stand me in good stead as I continue with my studies. Again, my thanks to all for the investment they have made in my personal and professional development.

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Chapter 1: Introduction

During neural development, individual neurons extend neurites making precise synaptic connections with other cells in the developing nervous system. Growing neurites are guided by structures called growth cones which sense directional cues in their microenvironment. In effect, growth cones act as sensors that detect and interpret signals which are present within the developing organism's nervous system. Significant experimental resources have been brought to bear on the question of how these small structures are able to sense and respond to cues as they grow. Cytologically, the behaviors exhibited by growth cones are relatively simple. These behaviors include turning towards attractive cues, and turning away from, or retracting in the presence of repulsive guidance cues. However, the signaling cascades within the growth cones which give rise to these behaviors are far more complex. Over the past several decades, significant strides have been made in understanding the sub-cellular mechanisms that give rise to such growth cone behaviors. While the signaling pathways show a common logic and degree of conservation, the number of factors capable of guiding neuronal growth cones is extensive, and novel guidance factors are frequently identified (Tessier-Lavigne and Goodman, 1996). One such factor, retinoic acid, a vitamin A derivative, has previously been shown to attract the neuronal growth cones of the mollusc *Lymnaea stagnalis* in culture (Dmetrichuk *et al.*, 2006, 2008). However, the sub-cellular mechanisms which are responsible for this attractive response have not been investigated in detail. The research which I report in this thesis addresses the sub-cellular signaling pathways within molluscan growth cones as they sense and respond to a gradient of retinoic acid (published in part in *Journal of Neuroscience*, Oct. 28, 2009).

By way of introduction, I survey the structure and basic capacity for motility possessed by the growth cone, the sub-cellular pathways known to be activated in response to guidance cues, a short overview of retinoic acid signaling, and finally the advantages of working with an invertebrate system.

1.01 The Growth Cone

The search for mechanisms which underlie the assembly of neural circuits within the embryonic brain has been a significant research question for developmental neurobiologists. Considering even a simpler nervous system, that of the mollusc *Aplysia* containing some 20 000 neurons, the task of neuronal assembly seems daunting (Williams and Herrup, 1988). Early insights into the process of network assembly came in 1880 when the neuroanatomist Ramon y Cajal characterized small, club-like expansions at the tips of growing neurites (de Castro *et al.*, 2007). Today, these structures are known as growth cones.

Structurally, the growth cone is a small cytoplasmic expansion found on the tips of developing or regenerating neurites. *In vivo*, growth cones take on a more compact, club-like shape, whereas *in vitro*, they appear more diffuse bearing some resemblance to an outstretched hand. In culture, invertebrate growth cones can be as large as 50µm in diameter (Lovell and Moroz, 2006). Histologically, the growth cone is described as possessing 3 regions: the C-, or central, domain, the P-, or peripheral domain and the T-, or transitional domain between the other 2 regions (Bouquet and Nothias, 2007) (Figure 1).

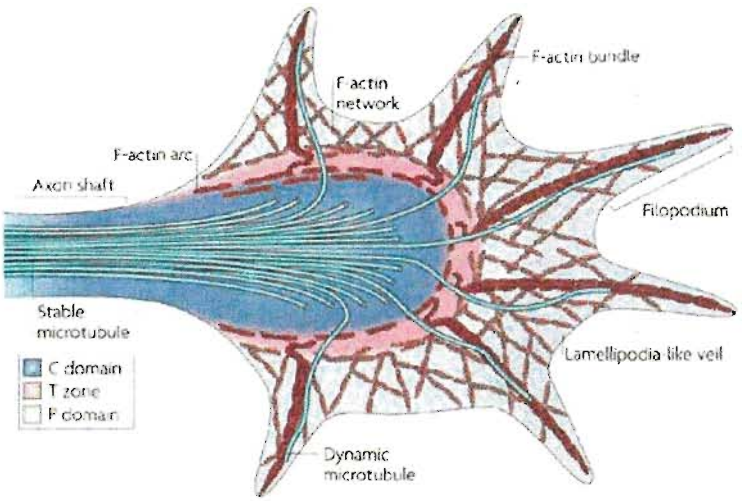


Figure 1. The Structure of the Growth Cone. The growth cone appears as an expansion of the cytoplasm at the tip of a growing neurite. It is histologically characterized as having three regions: The central (C)-domain where organelles and splayed microtubules are seen, the transitional (T)-domain where the actin arc and myosin motor proteins are concentrated and the peripheral (P)-domain where filopodia and lamellopodia are located. [from Lowery and Vacor, 2009]

A distinctive feature of the growth cone is the extension of long, slender, motile protrusions called filopodia. These structures are composed of an inner core of F-actin giving the filopodia their long, slender appearance. Embedded in the membrane are receptors for guidance. Filopodia are dynamic structures, extending and withdrawing, as the growth cone senses its environment. Between filopodial extensions are lamellipodia, veil-like protrusions rich in G-actin and short F-actin polymers which serve as a pool from which to draw as the growth cone continually modifies its peripheral structure. Both filopodia and lamellipodia are features of the P-domain (Bouquet and Nothias, 2007). The C-domain of the growth cone is its more robust structural domain containing organelles (e.g., mitochondria, ribosomes) and microtubules which provide structural support to the growth cone (Bouquet and Nothias, 2007). The T-domain is a

myosin-rich region separating the P- and C-domain. Myosin generates a contractile force on the P-domain's actin cytoskeleton leading to the formation of an actin arc within the growth cone which restricts the entry of microtubules into the P-domain (although microtubules are occasionally seen pushing down into the tips of filopodia) (Lowery and Vactor, 2009).

1.02 The Advancing Growth Cone

Within the developing brain, growth cones maneuver through a dense extracellular environment composed of proteins such as laminin and fibronectin, as well as various cell adhesion molecules, all of which promote the advance of growth cones. Yet, within this meshwork of extracellular matrix and neighboring cells exist both membrane-bound and diffusible guidance cues which direct the course of the growing neurite (Chilton, 2006; Wen and Zheng, 2006). Obviously then, the mechanisms responsible for forward movement of the growth cone must not only be able to engage and disengage, but also be biased in one direction or another, depending upon the input(s) received by the growth cone.

While at rest, the growth cone's motility machinery remains highly active. The continual myosin-dependent movement of F-actin from the filopodia back towards the T-domain occurs simultaneously with the polymerization of actin within the P-domain which also exerts some retrogradely directed force on F-actin which also drives it back towards the T-domain. This movement of F-actin, known as actin treadmilling, has been likened to an idling motor waiting to be engaged, hence the use of the term 'clutch

hypothesis' to describe growth cone motility (Kalil and Dent, 2005; Lowery and Vactor, 2009). When the growth cone encounters an adhesive substrate, membrane receptors bind the molecule leading to the formation of a molecular complex which functions to anchor the F-actin filaments to the bound receptor complex. This stops the retrograde flow of F-actin, while actin polymerization at the leading edge continues driving the growth cone forward (Lowery and Vactor, 2009) (Figure 2).

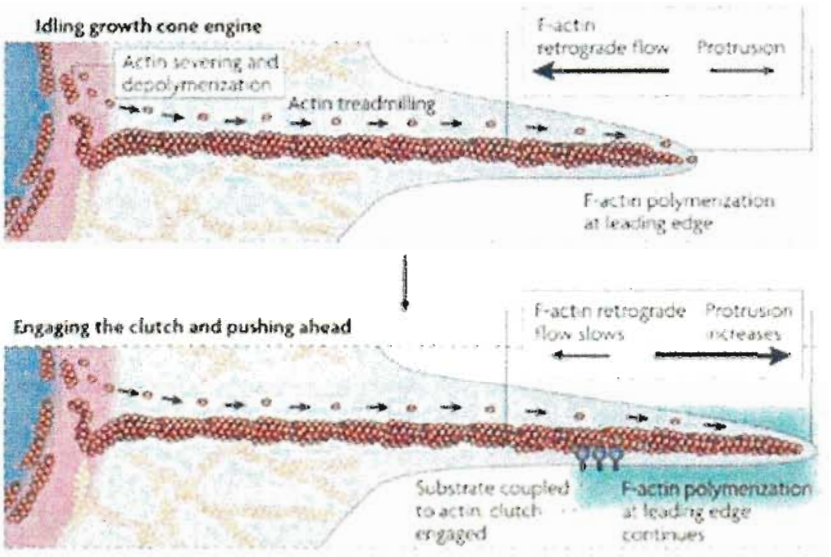


Figure 2. Schematic diagram showing the P-domain of the growth cone depicting the clutch hypothesis. Receptors which bind to the underlying substrate also bind F-actin essentially locking it in place. Polymerization at the distal end then drives the filopodia of the growth cone forward (from, Lowery and Factor, 2009).

Interestingly, it has been shown that the filopodia are the points of contact with the underlying substrate and the place where the force needed for forward growth is generated (Heidemann *et al.*, 1990). Other studies have also shown the essential need for filopodia in promoting normal growth (Dwivedy *et al.*, 2007).

The microtubules also play an important role in growth cone advance. Upon binding to an adhesive substrate, microtubules are seen 'searching' through the P-domain. The reason for this is not entirely clear. However, it has been suggested that they may play a role in guidance cue sensing by bringing factors attached to the microtubules into proximity with the activated membrane receptors. They may also participate in the formation of structural elements needed to process the signal received through the receptor (Lee *et al.*, 2007; Van Horck and Holt, 2008; Lowry and Vactor, 2009). Microtubules also consolidate the new axon which is being laid down as the growth cone advances (Goldberg, 2003). Disruption of microtubule dynamics inhibits turning responses in growth cones highlighting the significance of these structures in the turning apparatus (Buck and Zheng, 2002). Therefore, the cytoskeletal elements of the growth cone are essential for not only forward growth, but also for responding to environmental guidance cues.

1.03 Pathfinding and Guidance Molecules

The suggestion that chemical factors in the developing embryo guide growing neural processes was first proposed by Ramon y Cajal, and was initially known as the neurotropic hypothesis (Cajal, 1892; de Castro *et al.*, 2007). Ongoing research has shown Cajal's hypothesis to be remarkably accurate. The list of known, often highly conserved, guidance factors continues to expand. The best known guidance factors belong to the following families/receptors: netrins, DCC/UNCs (Serafini *et al.*, 1994; Kennedy *et al.*, 1994), semaphorins, neuropilins/plexins (Luo *et al.*, 1993), slits/Robos

(Tessier-Lavigne and Kidd, 1999; Kidd *et al.*, 1999; Brose *et al.*, 1999; Li *et al.*, 1999), and ephrins/Ephs (Drescher *et al.*, 1995). More recently, classical morphogenetic molecules (which are involved in morphogenesis in a concentration-dependent manner) including Wnts, TGFβ/BMPs, hedgehogs, and FGFs have been shown to be active later in development by guiding neural processes (Charron and Tessier-Lavigne, 2005). Neurotransmitters [e.g., serotonin (Haydon *et al.*, 1984), acetylcholine (Zheng *et al.*, 1994), glutamate (Zheng *et al.*, 1996), and dopamine (Spencer *et al.*, 2000a)] and neurotrophins [e.g., BDNF (Song *et al.*, 1997)] have also been shown to guide neuronal growth cones. Some evidence also point to biophysical phenomena, such as endogenous electric fields, playing a role in guidance, though such demonstrations have only been *in vitro* (Patel and Poo, 1982; McCaig *et al.*, 2005).

Guidance molecules are found throughout the embryonic nervous system as gradients which serve to attract or repulse growth cones. Some gradients are established by diffusible molecules (e.g., BDNF, netrin-1) while others are bound to a cell surface or to the extracellular matrix (e.g., ephrins/Ephs) (Bagnard, 2007). Recent studies have shown the remarkable sensitivity of individual growth cones in detecting gradients of guidance factors, being able detect a concentration difference of approximately 0.01% across the growth cone surface (Rosoff *et al.*, 2004). Even in the earliest stages of gradient detection, the growth cone is actively responding (see Mortimer *et al.*, 2007 for review). For example, when initially sensing an attractive GABA gradient, GABA receptors in the cell membrane are rapidly redistributed in a microtubule dependent manner to the side of the growth cone detecting the attractive

gradient (Bouzigues *et al.*, 2007). These changes occur before any significant ultrastructural changes are seen (e.g., filopodial extension).

An intriguing question concerns how the correct receptors are prevented from diffusing out of the growth cone membrane into other parts of the axon, or even up into the soma. In the axon, proximal to the growth cone, a region known as the axon initial segment (AIS) is present which acts as a diffusion impermeable barrier to membrane receptors in the growth cone/axon (Winckler *et al.*, 1999). Thus, while some movement within the membrane occurs, it is limited to the axonal compartment. The AIS appears to be composed of a cluster of transmembrane proteins anchored to the underlying cytoskeleton by proteins such as ankyrin (Allen and Chilton, 2009). It is also known that receptors within the growth cone itself can be anchored to the cytoskeleton, as well as existing in membrane associated complexes. However, significant mobility in these membrane receptors has also been noted (Bouzigues *et al.*, 2007). These findings illustrate that the membrane of the growth cone is a complex and highly regulated region which is essential for the precision with which growth cones sense and respond to their microenvironment.

1.04 Local Responses in the Growth Cone

The high level of autonomy possessed by growth cones has become increasingly clear over the past several decades. The first demonstration of this capacity came in 1987 when William Harris and colleagues demonstrated *in vivo* that the growth cones of *Xenopus* retinal ganglion cells would continue to travel towards the *Xenopus* optic

tectum even when transected from the cell body. Numerous other demonstrations of growth cones, mechanically isolated from their cell bodies, turning towards or away from a chemical gradients *in vitro* have been reported (Table 1).

Table 1. Examples of isolated neurites which retain their capacity to respond to chemical guidance cues.

Guidance Factor	Response	Cell Type	Reference
Engrailed-2	Attraction	*Xenopus* nasal retinal axons	Brunet *et al.*, 2005
Engrailed-2	Repulsion	*Xenopus* temporal retinal axons	Brunet *et al.*, 2005
Netrin-1	Repulsion	*Xenopus* retinal axons	Campbell & Holt, 2001
Sema3A	Collapse	*Xenopus* retinal axons	Campbell & Holt, 2001
Serotonin	Collapse	*Helisoma* B19 neurite	Davis *et al.*, 1992
Tectum, *in vivo*	Attraction	*Xenopus* retinal axons	Harris *et al.*, 1987

Growth cones are also known to store mRNA transcripts, and initiate local translation of these within the growth cone upon encountering certain guidance cues within its environment, or following traumatic injury (Willis and Twiss, 2006; Lin and Holt, 2007). Awareness of this capacity to initiate protein synthesis has precedents as far back as the early 1970s when polyribosomes were visualized within growth cones (Tennyson, 1970). It was not until the 1980s however that these histological findings began to draw attention (as similar discoveries were made at the base of dendritic spines) (Steward and Levy, 1982).

In a 2007 review article, Andrew Lin and Christine Holt discuss several possible reasons why local protein synthesis occurs within growth cones. First, previous research has shown that attractive signals (*e.g.*, netrin-1) lead to the synthesis of β-actin in intact

growth cones [Yao *et al.*, 2006]. One current hypothesis regarding attractive turning holds that the local synthesis of β-actin serves as a nucleation centre for the pre-existent G-actin resulting in polymerization. Therefore, the purpose of β-actin synthesis is not to generate a sufficient quantity of β-actin, but to create a nucleation centre from which to begin the cytoskeletal remodeling (Lin and Holt, 2007). It has also been suggested that the chaperone proteins, prefoldin and chaperonin, protect β-actin from taking intermediate forms during folding, which facilitates rapid polymerization by protecting against unwanted β-actin aggregation (Lin and Holt, 2007). Spatial efficiency may be another reason underlying local protein synthesis. Restricting mRNA transcripts to the distal tips of growth cones prevents cluttering of the cytoplasm in a way that storing proteins simply cannot (Lin and Holt, 2007).

1.05 Examples of Local Protein Synthesis

Though now dated, it was traditionally held that proteins which were required in the distal domains of neurons, the axon and dendrites, were synthesized in the cell body and shipped to these outer regions of the cell. However, it is now widely recognized that mRNA transcripts are localized to growth cones and axons in both developmental and regenerative contexts (Moccia *et al.*, 2003; Gioio *et al.*, 2004; Kindler *et al.*, 2005; Hengst and Jaffrey, 2007). In the vertebrate regenerative context, local translation is needed to respond to injury, and to reform a growth cone [Willis and Twiss, 2006; Zheng *et al.*, 2001; Verma *et al.*, 2005]. In rat sciatic nerve, neurofilament mRNAs are localized

to the axon and locally translated in response to axonal injury [Sotelo-Silveira *et al.*, 2000]. However, it is not only regeneration responses that require local synthesis.

Numerous studies have shown that local synthesis plays a developmental role as growth cones sense guidance cues in their microenvironments. For example, *Xenopus* growth cones responding *in vitro* to sema3A require the local synthesis of RhoA, a small GTPase involved in cytoskeletal rearrangement (Wu *et al.*, 2005). Also *Xenopus* retinal growth cones initiate the local synthesis of cofilin-1 in response to the repulsive cue, slit2. Cofilin-1 acts to destabilize F-actin leading to the collapse of the growth cone (Piper *et al.*, 2006). However, it is not only repulsive cues which require local protein synthesis. Attractive turning of *Xenopus* growth cones towards netrin-1 requires the local translation of β-actin, (Campbell and Holt, 2001; Leung *et al.*, 2006) as does turning towards a gradient of BDNF (Yao *et al.*, 2006). Attraction towards the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) also requires protein synthesis (Guirland *et al.*, 2003). Campbell and Holt (2001) have shown using radio-labeled amino acids that the initiation of local synthesis begins minutes after exposure to a guidance cue, with significant accumulation of radiolabeled protein occurring within 10 minutes. Interestingly, local protein degradation also appears necessary for responding to netrin-1, though not to Sema3A or slit2 (Campbell and Holt, 2001; Piper *et al.*, 2006).

Several studies have also demonstrated the local synthesis and insertion of functional receptors into the axonal membrane. Spencer *et al.* (2000b) showed that isolated

neurites can locally synthesize and insert functional receptors into the axonal membrane, and more recently Bi *et al.* (2006) showed the local synthesis of the κ-opioid receptor in the axons of DRG neurons. Britis *et al.* (2002) showed that neurons crossing the midline of the developing chick spinal cord initiate the local synthesis and insertion of the EphA2 receptor. This was the first demonstration of local synthesis *in vivo*. Studies to date, therefore, have shown that responding to certain guidance cues requires local protein synthesis or degradation (or both) of numerous proteins.

1.06 Calcium Influx in Growth Cone Responses

Calcium signaling is known to play remarkably diverse roles within cells. Within growth cones, calcium signaling is associated with changes in both morphology and motility. While global elevation in intracellular calcium in growth cones influences outgrowth, localized influx into growth cones creates calcium microgradients which influence the behavior of the growth cone (Henley and Poo, 2004). For example, when sensing an attractive guidance cue, the local elevation in calcium concentration on the side of the growth cone facing the guidance molecule causes an extension of filopodial and lamellipodial veils, and if sustained will lead to a reorientation of neurite outgrowth (Gomez and Zheng, 2006). Interestingly, while elevation of intracellular calcium can lead to extension of filopodia, it has also been observed to lead to the retraction of filopodia. This is taken to indicate that the nature of the calcium signal, its amplitude, frequency and source, play a key role in how the calcium signal is processed [Gomez and Zheng, 2006]. The importance of calcium signaling has been established for several

guidance cues including BDNF and netrin-1 [Song *et al.*, 1997; Hong *et al.*, 2000]. Calcium imaging has demonstrated that an internal gradient forms, with the highest concentration on the side of the growth cone sensing the gradient. However, even repulsive cues generate a calcium gradient, though the slope of the gradient is shallower than for attractive signals. Therefore, both the extent and slope of the calcium gradient together play an essential role in determining how the growth cone will respond [Gomez and Zheng, 2006]. The formation of these gradients is the result of both the opening of calcium channels in the growth cone membrane, and release of calcium from intracellular stores [Gomez and Zheng, 2006]. The downstream targets of the calcium signal are a matter of ongoing investigation, and depending on the response, the targets are likely not the same. It is thought that the calcium signal likely impinges on proteins involved in modeling and contracting the actin cytoskeleton. These would include proteins involved in bundling filamentous actin (α-actinin and fodrin) as well as proteins involved in destabilization of the cytoskeleton such as actin depolymerizing factor (ADF)/cofilin. It is also likely that calcium binds to calmodulin, which itself has a range of downstream targets including enzymes which function as kinases and phosphatases. There are also calcium-sensitive proteases, such as calpain, which are known to be involved in growth cone repulsion. Finally, calcium signaling is also known to affect the Rho GTPases, RhoA, Cdc42 and Rac, which play a role in remodeling the cytoskeleton [Henley and Poo, 2004; Gomez and Zheng, 2006]. Taken together, the importance of calcium signaling in growth cone behavior is clear; however, the precise mechanisms

through which calcium signals brings about changes in growth cone morphology and growth remain less certain.

1.07 Retinoic acid

One of the chemotropic molecules to which growth cones are known to respond is retinoic acid, though the mechanism generating this response has yet to be determined. Retinoic acid is a small lipophilic molecule (300.4Da) which is derived from retinol (vitamin A). Because retinol cannot be synthesized with the complement of metabolic enzymes possessed by animals, it must be obtained through dietary means, often by feeding on plants, which serve as a source of carotenoids, from which retinal can be derived (Maden and Hind, 2003). Retinol can be metabolized into both retinal and retinoic acid. This conversion occurs inside the cell, with retinol being converted to retinal by *retinol dehydrogenases* and then to retinoic acid by *retinaldehyde dehydrogenases* (Maden and Hind, 2003). Retinoic acid can exist in numerous isoforms, two of which, *all-trans* and *9-cis* retinoic acid, are known to be biologically active in the nervous system of the model organism used in this study (*Lymnaea stagnalis*) (Dmetrichuk *et al.*, 2008) (see Appendix 1). The cytochrome P450 enzyme, CYP26, regulates retinoic acid levels following its synthesis by metabolizing retinoic acid to numerous products, which are then cleared from the cell (Maden and Hind, 2003) (Figure 3).

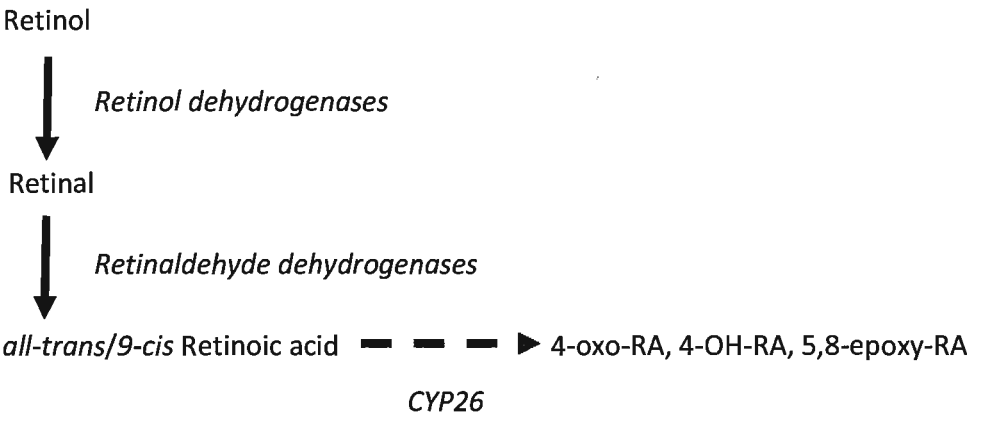


Figure 3. Schematic showing the synthesis and metabolism of retinoic acid.

Retinoic acid binds to ligand-activated transcription factors that belong to the steroid superfamily of hormone receptors, of which two major families exist: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Studies with the vertebrate receptors have shown that *all-trans* retinoic acid binds with high affinity to the RARs, but the RARs are also activated by *9-cis* retinoic acid. In contrast, RXRs are activated 10-100 times more strongly by the *9-cis* isomer (Umemiya *et al.*, 1997). Once activated the receptors form a heterodimer (RAR-RXR, though RXRs can also form homodimers), which binds to specific DNA sequences, retinoic acid response elements (RAREs), which ultimately induce novel gene transcription. RAREs are definitively associated with 27 genes, though upwards of 500 genes have been shown to be affected by retinoic acid signaling (Maden, 2007). However, there is some evidence for non-genomic activity of RARs, including modulation of synaptic transmission and translational regulation of dendritically localized mRNA transcripts (Liao *et al.*, 2004; Poon and Chen, 2008). There is also evidence that retinoic acid may interact with molecules other than its classical

receptors. For example, PKCα has a binding site for retinoic acid, and is likely modulated by it (Ochoa *et al.*, 2003). Nevertheless, classically, retinoic acid has been thought to exert genomic effects in cells.

Studies in vertebrate organisms have revealed two major areas of neurodevelopment to which retinoic acid signaling contributes: neuronal patterning and neuronal differentiation. For example, along the anteroposterior axis of the neural tube, retinoic acid acts synergistically with the classical morphogen molecules Wnts and FGFs to organize the posterior hindbrain and anterior aspect of the spinal cord (Maden, 2007). Retinoic acid appears to play a crucial role in this aspect of neurodevelopment, as the abolition of its signal prevents the formation of the posterior hindbrain and introduces defects into the anterior spinal cord. The gradient of retinoic acid is established by its synthesis in the posterior mesoderm and catabolism in the anterior mesoderm by CYP26C1 (Maden *et al.*, 1996; Glover *et al.*, 2006). Retinoic acid also plays a role in patterning the dorsoventral axis of the neural tube in the developing chick embryo (along with the morphogens SHH, BMPs and FGFs) (Maden, 2007). Together these morphogens specify cell fates of sensory, motor and interneurons within the developing spinal cord (Maden, 2007). The second functional role of retinoic acid, neuronal differentiation, has been extensively studied in embryonal carcinoma cells and neuroblastoma cells, where it has been shown to contribute to both neuronal and glial cell specification (Maden, 2007). Acting in this capacity, retinoic acid leads to the activation of many types of genes including transcription factors, cell signaling

molecules, structural proteins, enzymes and cell surface receptors (Maden, 2007). Retinoic acid has also been shown to regulate differentiation *in vivo*. In the chick embryo, both the somatic motor neurons of the hindbrain region and the lateral motor columns in the spinal cord are developmentally regulated by retinoic acid signaling, both in terms of number and cell type. Interestingly, in the absence of retinoic acid, developing spinal neurons fail to extend neuronal processes, pointing to a role for retinoic acid in neurite outgrowth *in vivo* (Maden, 2007). There is additional evidence that retinoic acid promotes the outgrowth of neuronal processes. For example, retinoic acid has been shown to potentiate outgrowth from several tissues *in vitro*, including embryonic spinal cord and neural tube (Wuarin *et al.*, 1990; Maden *et al.*, 1998), dorsal root ganglia (Corcoran *et al.*, 2000), cerebellar tissue (Yamamoto *et al.*, 1996), and sympathetic ganglia (Plum *et al.*, 2001). Both *all-trans* and *9-cis* retinoic acid promote neurite outgrowth in human neuroblastoma cells (Han *et al.*, 1995). Cultured spinal cord explants from the newt, *Notophthalmus viridescens*, also showed outgrowth which was directed towards a retinoic acid-soaked bead (Dmetrichuk *et al.*, 2005). Interestingly, cells cultured from embryonic chick neural tube tissue also exhibited *directed* outgrowth towards a source of retinoic acid (Maden *et al.*, 1998). These latter findings suggest that retinoic acid may also possess *chemotropic* effects, being able to guide neurite outgrowth.

While less is known about retinoic acid signaling in invertebrates, there is also evidence that retinoic acid signaling plays an *in vivo* role in invertebrate regeneration

and development. For example, in the Fiddler crab, *Uca pugilator*, retinoic acid signaling is known to play a role in limb regeneration [Hopkins, 2001]. Furthermore, the RXR is known to be present in the fiddler crab, and endogenous *9-cis* retinoic acid has been detected in the crab's limb blastema (Hopkins *et al.*, 2008). The RXR has also been shown to be expressed in the gastropod mollusc, *Thais clavigera* (Horiguchi *et al.*, 2007). *9-cis* retinoic acid, signaling through the RXR, has been shown to play a role in the development of the penis and vas deferens of *T. clavigera* (Horiguchi *et al.*, 2008).

Recently, Dmetrichuk *et al.* (2008) using high-pressure liquid chromatography showed that both *all-trans* and *9-cis* retinoic acid were present in the CNS of *Lymnaea stagnalis*. Earlier work had shown that *all-trans* retinoic acid was able to stimulate neurite outgrowth from cultured *Lymnaea* visceral F neurons (Dmetrichuk *et al.*, 2006). This observation may suggest the evolutionary conservation of retinoic acid-induced outgrowth. This would not be an unusual finding as many trophic or tropic factors which function in vertebrates also do so in invertebrates (Tessier-Lavigne and Goodman, 1996). Dmetrichuk *et al.* (2006) also showed that retinoic acid had a tropic effect on molluscan neuronal processes by attracting the growth cones towards a gradient of *all-trans* retinoic acid. In addition, the attractive effect was maintained for up to 24 hours [Dmetrichuk *et al.*, 2006]. This was the *first* demonstration of *all-trans* retinoic acid producing growth cone turning in an *invertebrate system*. Additionally, a gradient of *9-cis* retinoic acid was also shown to attract *Lymnaea* growth cones (Dmetrichuk *et al.*,

2008). However, despite these important findings, the sub-cellular mechanisms mediating this chemoattraction remained largely unexamined.

Also of significance, for the purpose of this study, is the fact that an RXR with 80% homology to the vertebrate RXRα has been cloned from *Lymnaea stagnalis*, as has the retinoic acid synthesizing enzyme, *retinal dehydrogenase* (Carter *et al.*, 2006). The RXR and *retinal dehydrogenase* proteins have also been shown to be expressed in the CNS of *Lymnaea* (Cater *et al.*, 2006). Incubation of *Lymnaea* embryos in the presence of an RXR pan-agonist also led to significant morphological defects (C. Carter and G.E. Spencer, personal communication). In summary, while the understanding of retinoic acid signaling in invertebrates is less advanced than in vertebrates, it is clear that proteins involved in retinoic acid signaling are present in at least some invertebrates, and that retinoic acid potentiates neural outgrowth and acts as a chemoattractant to growth cones in molluscan neurons. Therefore, retinoic acid appears to be an important molecule for both vertebrate and invertebrate development and regeneration.

1.08 Molluscan model systems & *Lymnaea stagnalis*

Experiments with invertebrate animals have made important contributions to neurobiology. Molluscs have proven to be an especially good model organism for neuroscientists. *Aplysia californica*, *Helisoma trivolvis* and *Lymnaea stagnalis* are the most frequently used molluscan species. (Work has also been done in squid giant axons). There are a number of reasons for the success of research in these organisms.

First, molluscan neurons are large and identifiable, making it feasible to work with individual cells. Second, these neurons can also be isolated from ganglia and grown in culture, as adult neurons regenerate neuritic processes. Third, many of the neurons are well characterized in terms of their function. Finally, in culture these neurons extend long, extensively branching processes which are tipped with large growth cones (Lovell and Moroz, 2006). In this study, the mollusc *Lymnaea stagnalis* was used. Cultured neurons from this organism have been used previously to study processes such as neurite outgrowth, growth cone dynamics and neuronal regeneration (Syed *et al.*, 1990; Dmetrichuk *et al.*, 2006, 2008). These previous experiments also perfected an effective cell culture system for these *Lymnaea* neurons, which utilized in this study. Following their isolation from intact ganglia, the processes begin to regenerate within 10-18 hours, often producing extensive outgrowth in culture, and continuing to grow for up to 72 hours. Furthermore, these molluscan neurites can be mechanically isolated from the cell body and remain viable *in vivo* for up to 48 hours (van Kesteren *et al.*, 2006). This feature makes these neurons especially useful in studying growth cone dynamics since it allows local responses in the growth cones to be effectively assessed. Furthermore, as noted above, the large size of the growth cones allows for *in vitro* manipulations to be performed with greater ease than can be done using the much smaller vertebrate neurons. The larger size also permits subtle changes in the growth cone to be detected. For example, the earliest stages of chemoattraction involve the reorientation of filopodia on the side of the growth cone facing the gradient. Under the microscope, such changes can be clearly seen and photographed using these molluscan neurons.

These features make the mollusc, *Lymnaea stagnalis*, an excellent model system in which to conduct experiments on neuronal growth cones.

The general objective of this thesis is to elucidate the signaling pathways which are activated within the growth cone by a gradient of *all-trans* retinoic acid. Based on previous knowledge of growth cone behavior, I hypothesized that a local mechanism involving protein synthesis and calcium influx is involved. I thus aimed to determine whether the retinoic acid-induced growth cone turning required local protein synthesis and/or calcium influx, and also assessed the potential role of several proteins which have previously been shown to be involved in retinoic acid signaling. My overall findings support a novel, non-genomic signaling pathway for the chemotropic actions of *all-trans* retinoic acid in *Lymnaea* pedal A growth cones.

Chapter 2: Materials and Methods

1.01 Animals

The pond snail, *Lymnaea stagnalis* (Figure 4A), was used for all experimental work in this study. *Lymnaea* were housed in open air tanks containing artificial, aerated water (Instant Ocean Sea Salt, 5g/L) and were fed with romaine lettuce and fish food daily (Nutrafin Max Spirulina Algae Flake Food). All animals used for cell culture work, ranged in size from 20 to 25mm (approximately 10 weeks after emerging from the egg sacks).

2.02 Cell Culture

Individual CNSs were dissected from anesthetized (10% Listerine in saline) snails. CNSs were passed through a series of 6 antibiotic rinses (gentamycin (4.2×10^{-5}M) Sigma-Aldrich), treated with trypsin for 22 minutes (6mg in 3mL DM (defined media), Sigma-Aldrich), and followed by soybean trypsin inhibitor for 12 minutes (6mg in 3mL DM, Sigma-Aldrich). CNSs were then pinned out in a dissection dish containing high osmolarity DM (750µL of 1M glucose in 3mL DM), and the outer connective tissue, and inner sheathing around the left and right pedal ganglia were removed (Figure 4B).

Individual pedal A neurons were removed from the ganglia using a fire-polished glass pipette (approximately 50-60µm in diameter). Suction was applied using a microsyringe, which drew individual cells into the pipette, severing their axons (Figure 5A, B). Individuals cells were then placed into poly-L lysine coated culture dishes containing 3mL of conditioned media (CM) (see below for details). Dishes were supplemented with *all-trans* retinoic acid which is known to promote outgrowth from cultured neurons (10^{-7}M final bath concentration; 0.001% ethanol (EtOH) vehicle in

bath). Extensive outgrowth was seen within 18 to 20 hours (*cf.* Figure 5C). This protocol has been successfully utilized in this system previously (Syed *et al.*, 1990; Hamakawa *et al.*, 1999; Dmetrichuk *et al.*, 2006, 2008).

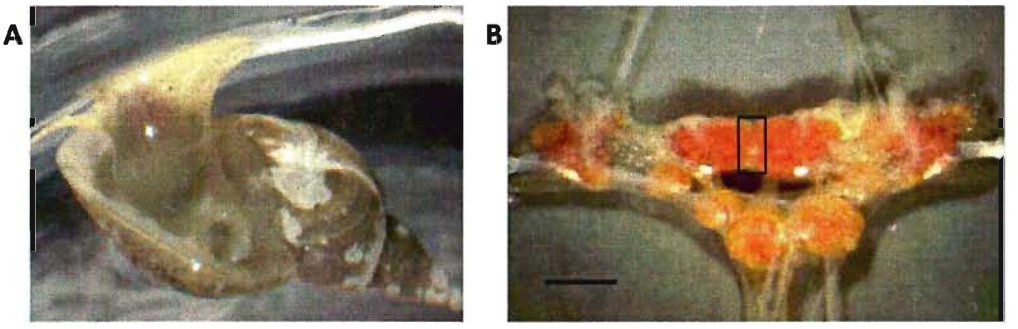


Figure 4. Preparation of *Lymnaea* CNS for neuron extraction. **A.** *Lymnaea stagnalis*. **B.** *Lymnaea* CNS pinned out in dissection dish (dorsal surface upward). The connective tissues have been removed from the CNS. The black box indicates the region containing the pedal A neurons. Scale bar, 1000μm

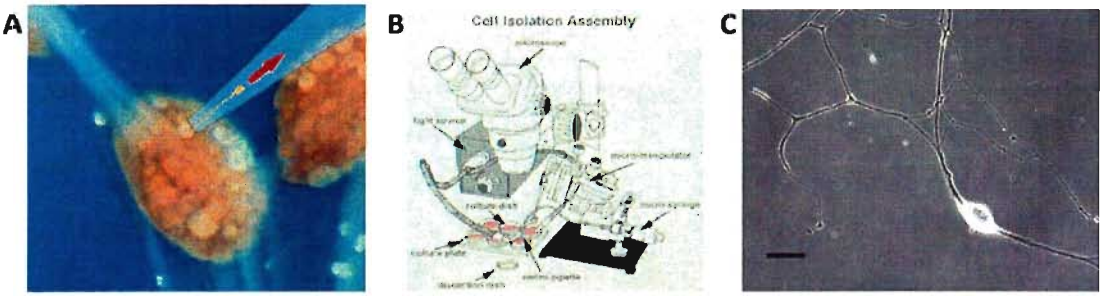


Figure 5. Neuronal cells pulled from the pedal ganglia are grown up in culture to produce extensive outgrowth. A. Suction applied to a fire polished glass pipette is used to draw up individual pedal A neurons which can then be placed into culture plates containing growth medium. **B.** Schematic showing the apparatus and setup used to remove individual cells from the *Lymnaea* CNS. Images A and B, courtesy of N. Syed. **C.** Extensive outgrowth generated from a pedal A neuron after 48 hours in CM, scale bar, 30μm.

CM was prepared by incubating dissected *Lymnaea* CNSs in DM for intervals of 4 days for up to 16 days in total. CM incubated with CNSs from the first 4 days (1x) was discarded. CM from each subsequent 4 day incubation up to a maximum of 16 days (2x, 3x, 4x) was used to culture neurons, as CM beyond this, fails to promote outgrowth.

Table 2. Incubation timetable for the production of conditioned medium (CM) for cell culture.

Days	CM
0-4	1x, discarded
4-8	2x, used for culture
8-12	3x, used for culture
12-16	4x, used for culture

Between 10 and 12 CNSs were incubated in 7mL of DM. All neuronal cell culture work was done with 2, 3 or 4x CM. CM contains unidentified trophic factors that are essential for generating outgrowth *in vitro*. Prior to incubating CNSs in DM, they were passed through a series of 20 antibiotic rinses containing gentamycin [4.2×10^{-5}M] (Sigma-Aldrich). This was repeated prior to each incubation period in DM. All CM was checked for contamination using an inverted microscope (Zeiss Axiovert 200, 40x) and was discarded if bacterial contamination was found.

Cell culture dishes (Falcon Easy Grip Petri dishes, #351008) were prepared by drilling a hole (1cm in diameter) through the dish, and gluing a glass cover slip below the hole. Plates were then sterilized with 95% EtOH and allowed to dry overnight, after which they were coated with poly L lysine for 30 minutes (1mg/mL Tris buffer). Finally, three 15 minute rinses with autoclaved, distilled water were performed. CM and cultured

cells were then added to the plates. The cultured cells were placed into the cover slip area of the dish, as this was the area which was coated with poly-L lysine. Cells were subsequently visualized under the microscope.

2.03 Growth Cone Turning Assays

After 18 to 20 hours in culture, many neurons had growing processes tipped with active growth cones. Individual growth cones were photographed over the course of 1 hour, allowing outgrowth speeds to be determined (μm/min), and to ensure the presence of sustained growth and a steady trajectory. The average rate of outgrowth for pedal A neurons in CM was 0.702 ± 0.459μm/min (n=10). This photographic procedure was also done in the presence of each inhibitor to ensure these agents did not disrupt neurite outgrowth (see Appendix 2 for a summary table). No inhibitor was found to disrupt neurite outgrowth ($p>0.05$, 1-way ANOVA). A gradient of *all-trans* retinoic acid (10^{-5}M in pipette) was applied to individual growth cones with a pressure pipette (Eppendorf-Femtojet, 4-8μm). Pressure application of DM alone has previously been shown not to attract *Lymnaea* growth cones (Dmetrichuk *et al.*, 2006), so this control was not repeated in this study. The pressure pipette was placed between 50 and 150μm from the growth cone, depending on the size of the pipette's opening (range, 4-8μm). Application pressure varied slightly depending on the size of the pressure pipette and distance from the growth cone, with application pressures of 5-12hPa used. Holding pressures of 1-2hPa were used to prevent backflow into the pipette between applications. Previous work with pipette assays has shown that the

concentration of the agent in the pipette is 100-1000 times less when it reaches the growth cone (Lohof *et al.*, 1992). Therefore, growth cones are likely exposed to concentrations of *all-trans* retinoic acid between 10^{-7} and 10^{-8}. Experiments lasted a minimum of 1 hour, while control experiments lasted at least 45 minutes, with turning assessed throughout the experimental course.

2.04 Isolation of neurites

Isolated neurites were mechanically separated from the soma using a sharp glass electrode (Figure 6A, B). This resulted in a complete separation of the neurite from the cell body, and monitoring continued throughout the course of an experiment to ensure that contact had not been reestablished (Figure 6C).

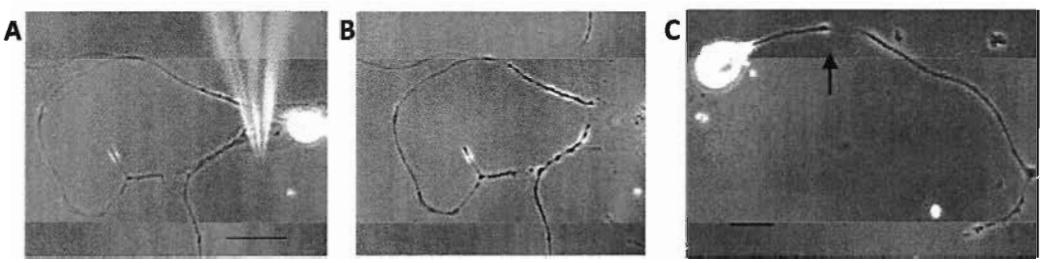


Figure 6. Neurites mechanically isolated from their cell body using a sharp glass electrode. A. The intact neurite was severed near the cell body. Scale bar, 60μm. **B.** The cell body of the neuron was completely removed from the dish leaving only the isolated neurites. (Images A and B from Farrar *et al.*, 2009; courtesy, J. Dmetrichuk). **C.** The cell body was not removed in every case. When left in the dish, the cell was checked at regular intervals to ensure the separation remained complete. The black arrow indicates the transection site which was monitored throughout the experiment. Scale bar, 30μm

Isolated *Lymnaea* neurites continued to grow following transection as shown previously (van Kesteren *et al.*, 2006). After isolation, neurites were given 15 minutes to recover

from the trauma before beginning the growth cone turning assay. Neurites which halted their growth or collapsed by completely retracting their growth cone were not used in the experiments. Isolated neurites which remained viable were exposed to a gradient of *all-trans* retinoic acid in the same manner as intact neurites.

2.05 Chemicals

All-trans retinoic acid (Sigma-Aldrich) was prepared in absolute EtOH and diluted to a final concentration of 10^{-5}M using DM (0.1% final EtOH concentration). The control solution was 0.1% EtOH in DM in the pipette. Anisomycin (Sigma-Aldrich)(4.5×10^{-5}M, bath concentration), which was used as an inhibitor of protein synthesis, was prepared in sterile distilled H_2O (Feng *et al.*, 1997). Gö6976 (Sigma-Aldrich), an inhibitor of protein kinase C (PKC), was prepared in dimethyl sulfoxide (DMSO) and diluted in DM to a final bath concentration of 10^{-5}M (Lacchini *et al.*, 2006) . Control experiments with 0.1% DMSO in the bath were performed. To disrupt calcium influx, cadmium (Sigma-Aldrich) was used. Cadmium was dissolved in sterile, distilled H_2O and diluted in DM to a final bath concentration of 10^{-5}M (Dmetrichuk *et al.*, 2008). The RXR pan-antagonist, PA452, was dissolved in DMSO and diluted to a final bath concentration of 10^{-6}M. The RXR pan-agonist, PA024, was also dissolved in DMSO and diluted to a concentration of 10^{-5}M in the pipette. PA452 and PA024 were both kind gifts of Dr. H. Kagechika, Japan.

2.06 Immunostaining

A custom made antibody against a synthetic peptide from the predicted 'hinge' region of the *Lymnaea* RXR covering the amino acid residues 183-198 between the DNA

binding domain and ligand binding domain. This custom made LymRXR was produced in New Zealand white rabbits and affinity purified from the antisera by Pacific Immunology Corp. (Ramone, CA). This antibody was used for the immunostaining of the Pedal A cultured neurons which was performed by C. Carter according to methodology described in detail in Carter et al., (2010).

2.07 Analysis

All images were captured using a Zeiss Axiovert 200 inverted microscope, Q imaging Retiga Exi camera and Northern Eclipse software (Empix Imaging, ON). Individual turning angles were determined by measuring the angle between the growth cone's initial trajectory and the maximum angle of turning observed during the course of the experiment. Approximately 30 images were taken over the course of each growth cone turning assay. An attractive turning response had a positive angle, and the growth cone turned towards the pipette, whereas a repulsive response had a negative angle, and the growth cone turned away from the pipette. All statistical analysis was performed using SigmaStat software. A one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* test was performed on all data obtained with intact neurites, though these data are presented separately throughout the results section. Likewise, all experimental data on isolated neurites were analyzed together with a one-way ANOVA and the Tukey-Kramer *post-hoc* test. Any other comparisons made are indicated separately in the text. Data are expressed as mean ± standard error of the mean (S.E.M.).

Chapter 3: Results

Previous work by Dmetrichuk *et al.* (2006) has demonstrated that cultured *Lymnaea* visceral F neurons are attracted to a focally applied gradient of *all-trans* retinoic acid. In this study, I elected to use *Lymnaea* Pedal A (PeA) motoneurons as they produce more extensive outgrowth in culture than the VF neurons. It was, therefore, necessary to first establish that cultured PeA neurites were also chemotactically attracted to a gradient of *all-trans* retinoic acid.

3.01 PeA neurites turn towards a gradient of *all-trans* retinoic acid

Following a period of 18-20 hours of culture in CM supplemented with *all-trans* retinoic acid (10^{-7}M, final concentration), active neurite outgrowth was present. Prior to application of *all-trans* retinoic acid to the growth cone, the trajectory was monitored to ensure that no abrupt changes in its course were seen. *All-trans* retinoic acid (10^{-5}M) was then applied to individual growth cones using a pressure pipette held at an angle to the advancing growth cone. The position of the pipette was adjusted throughout the experiment as the neurite advanced. PeA neurites were found to turn towards an applied gradient of *all-trans* retinoic acid with an average angle of 61.5° ± 11.6° (n=10) (Figure 7Ai-iv). A histogram showing the maximum turning angle for each growth cone is shown in Figure 8A. As ethanol (EtOH) was used as the vehicle for *all-trans* retinoic acid, control experiments were conducted with EtOH in the pipette (0.1% EtOH in DM). Analysis revealed that PeA neurites were not attracted towards a gradient of EtOH, showing an average turning angle of -6.4° ± 7.2° (n=10) (Figure 7Bi-iv). A Histogram showing the maximum turning angle for each growth cone in response to EtOH is shown

in Figure 8B. There was a significant difference between the growth cone turning angle towards *all-trans* retinoic acid in CM compared to the growth cone turning angle in response to EtOH ($p<0.001$) (Figure 8C). Comparison of the responses of VF growth cones to *all-trans* retinoic acid published previously (Dmetrichuk *et al.*, 2006) with the PeA growth cones of this study failed to show any significant difference (t-test, $p=0.759$, n.s.). This is the second cell type in which *all-trans* retinoic acid-induced chemoattraction has been demonstrated.

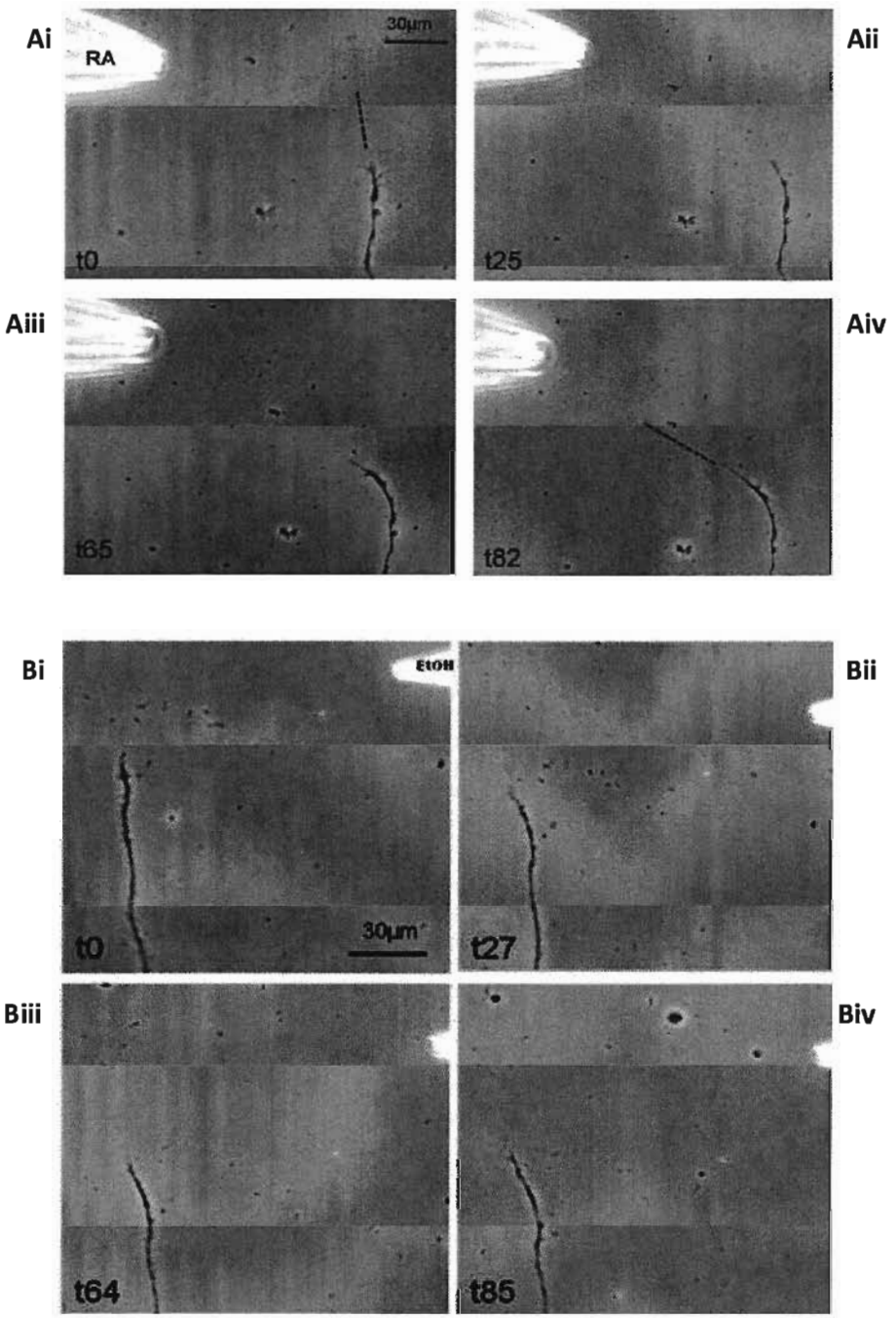


Figure 7. Intact Pedal A growth cones were attracted to a gradient of *all-trans* retinoic acid. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid. Dashed lines approximate beginning and end trajectories for each growth cone. Times (t) are given in minutes. **Bi-iv.** Growth cones are not attracted to a gradient of EtOH.

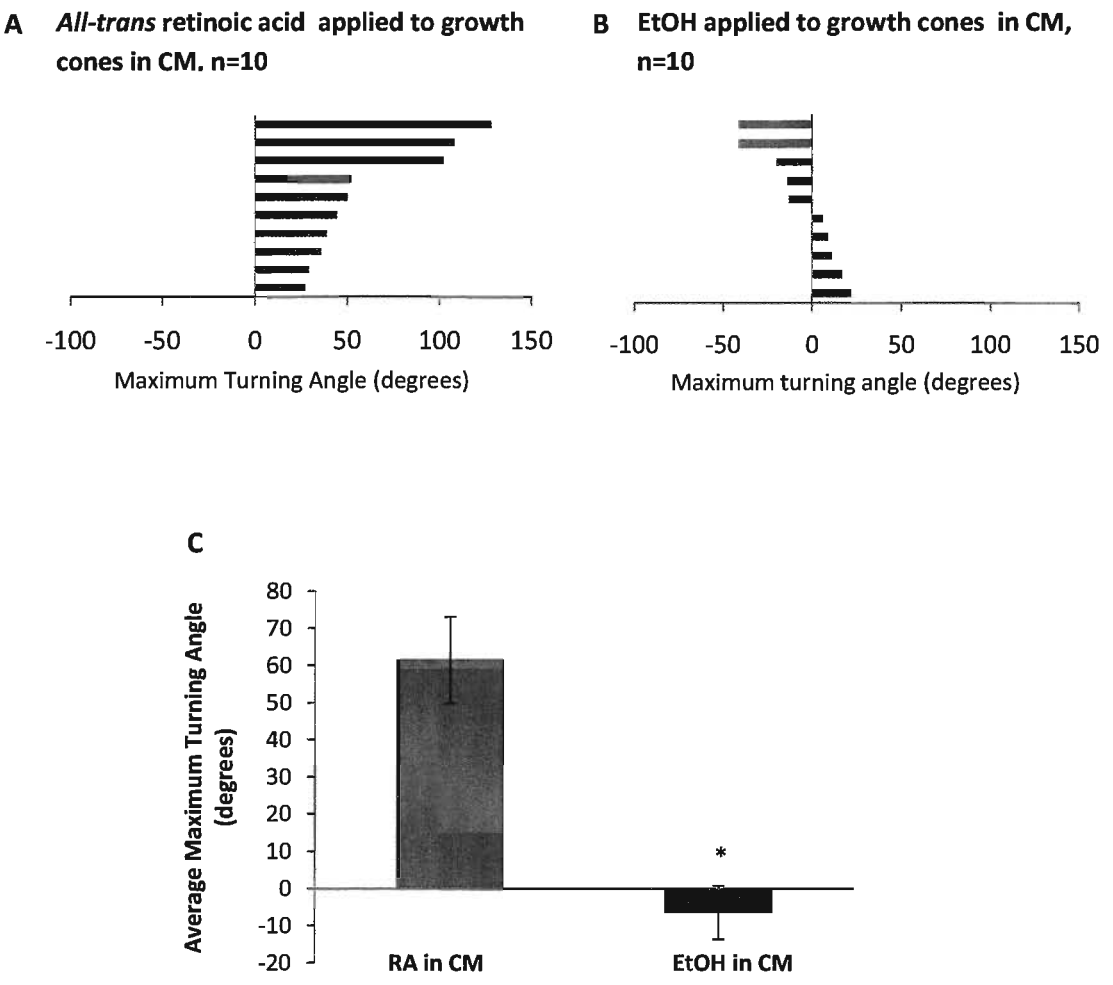


Figure 8. Intact Pedal A growth cones were attracted to a gradient of *all-trans* retinoic acid, but not ethanol. **A.** Histogram showing the maximal turning angle for each growth cone in response to a gradient of *all-trans* retinoic acid. Each bar represents the response from one growth cone. Positive turns are towards the pipette; negative turns are away from it. **B.** Histogram showing the maximal turning angle for each growth cone in response to a gradient of EtOH. **C.** Summary graph showing the average maximal turning angle exhibited by the growth cones in response to *all-trans* retinoic acid and EtOH. A statistically significant difference is present between the two conditions,*, $p<0.05$. Error bars represent the standard error of the mean (S.E.M.).

3.02 Isolated PeA neurites turn towards a gradient of *all-trans* retinoic acid

Classically, *all-trans* RA has been shown to signal through nuclear receptors, initiating gene transcription. However, previous work has shown that both VF and PeA neurons retain their ability to turn towards a gradient of *all-trans* retinoic acid when in the presence of the transcription inhibitor actinomycin D (Dmetrichuk, 2007; Farrar *et al.*, 2009). A final bath concentration of 50μM actinomycin D was used, as this has been shown to be effective in *Aplysia* cell culture (Lyles *et al.*, 2006); however, lower concentrations have also been used effectively in *Lymnaea* (Hamakawa *et al.*, 1999). Given that some growth cone responses are local (*cf.* Table 1), this transcriptional independence suggested the *possibility* of a local mechanism mediating the response to *all-trans* retinoic acid. To determine if this was the case, a series of experiments with neurites isolated from their cell bodies was performed. If isolated neurites retained the ability to respond to a gradient of *all-trans* retinoic acid, this would be strong evidence that a local mechanism was responsible for the response.

To determine whether a local response was involved, I took advantage of the previously established finding that mechanically isolated molluscan neurites can survive and continue to grow in culture for up to 24 hours (van Kesteren *et al.*, 2006). Following 18-20 hours in culture, long, fast growing neurites were mechanically isolated using a sharp glass pipette. The separation from the cell body was total, so that no communication with the cell body could occur. Upon exposure to a gradient of *all-trans* retinoic acid (10^{-5}M), isolated neurites exhibited an chemoattractive response showing

an average turning angle of 40.5° ± 7.4° (n=10) (Figure 9Ai-iv). The histogram showing maximum turning angles for each isolated neurite is shown in Figure 10A. Isolated neurites were not attracted to a gradient of EtOH, showing an average turning angle of -2.5° ± 7° (n=7) (Figure 9Bi-iv, 10B). Comparison of the growth cone turning responses towards *all-trans* retinoic acid and EtOH in isolated neurites found a significant difference between the conditions ($p<0.001$) (Figure 10C). Furthermore, no difference in turning angle was found between intact and isolated neurites in response to a gradient of *all-trans* retinoic acid (t-test, $p=0.144$, n.s.). Therefore, growth cones isolated from the cell body retain the ability to turn towards a gradient of retinoic acid, showing that *all-trans* retinoic acid is signaling through a local, non-genomic mechanism.

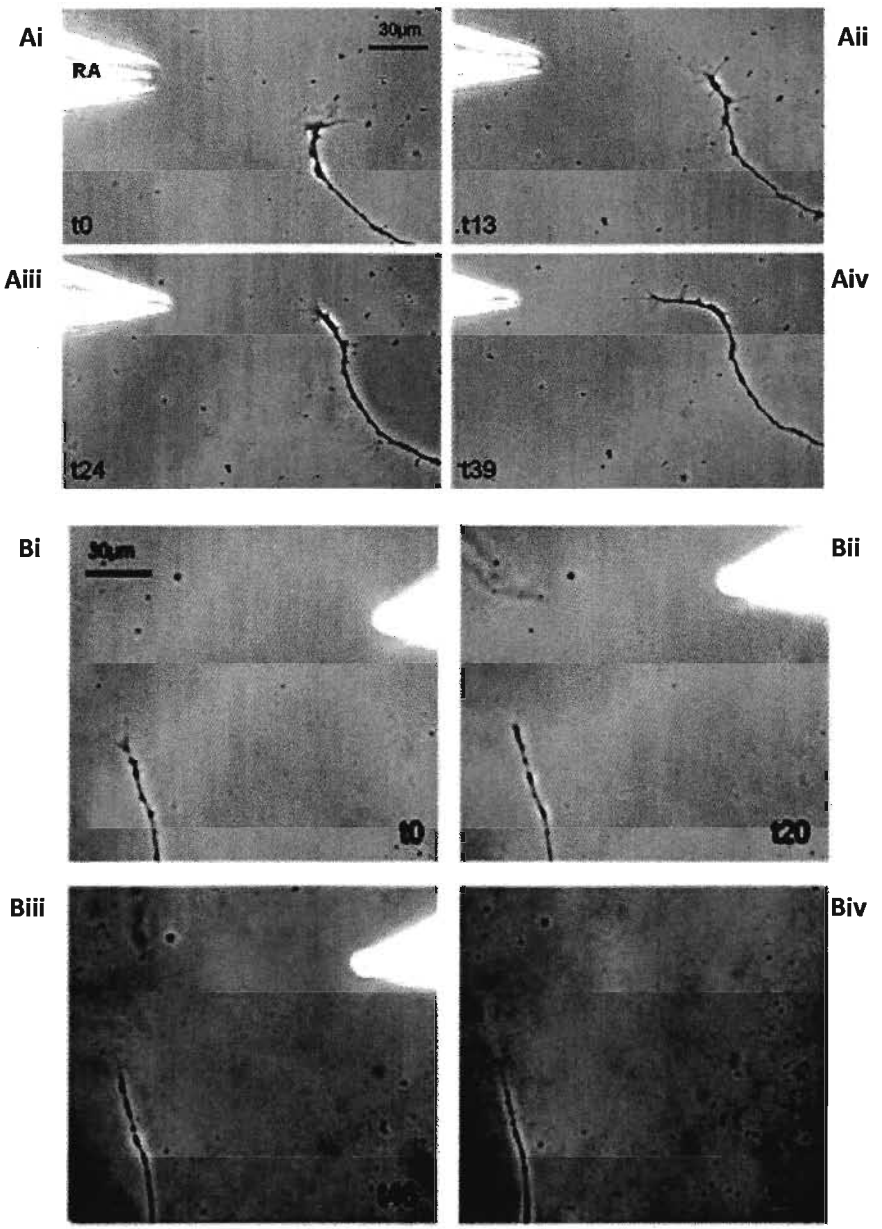


Figure 9. Isolated neurites turn towards a gradient of *all-trans* retinoic acid. Ai-iv. Representative micrographs show the turning course of an isolated growth cone during exposure to a gradient of *all-trans* retinoic acid. **Bi-iv**. Isolated growth cones are not attracted to a gradient of EtOH. Times are given in minutes (t).

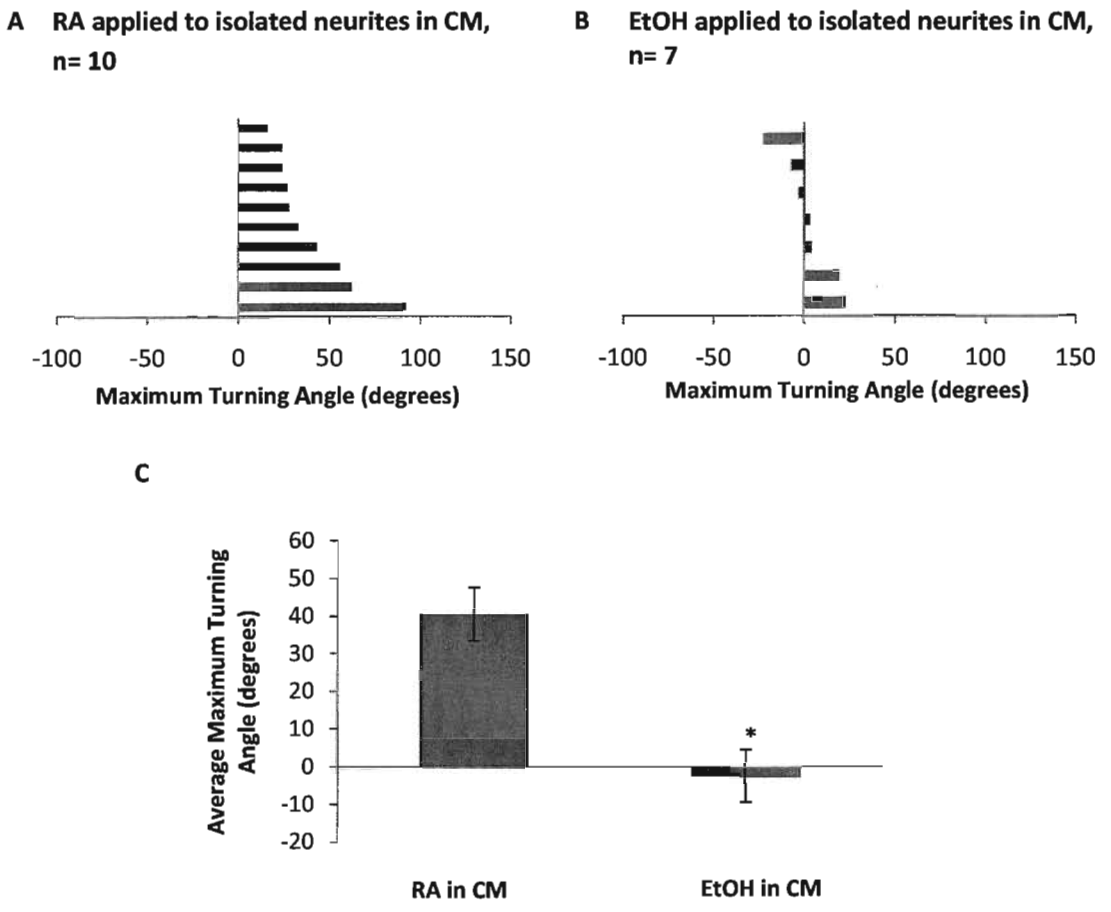


Figure 10. Isolated neurites are attracted to a gradient of *all-trans* retinoic acid, but not to EtOH. A. Histogram showing the maximum turning angle for each isolated neurite exposed to a gradient of *all-trans* retinoic acid. Each bar represents the response from one growth cone. Positive turns are towards the pipette, negative turns away from it. **B.** Histogram showing the maximum turning angle for each isolated neurite exposed to a gradient of EtOH. **C.** Summary graph showing the average maximal turning angle exhibited by the growth cones in response to *all-trans* retinoic acid and EtOH. A statistically significant difference is present between the two conditions, *, $p<0.05$. Error bars represent the standard error of the mean (S.E.M.).

3.03 Turning towards *all-trans* retinoic acid requires protein synthesis

Previous research has shown that responses to certain guidance cues depend on *de novo* protein synthesis (Lin and Holt, 2007). However, this is not the case for all guidance cues (Roche *et al.*, 2009). I was, therefore, interested in determining whether the response to *all-trans* retinoic acid was dependent on protein synthesis. Anisomycin has been previously used to inhibit protein synthesis in growth cone experiments (Campbell and Holt, 2001) and has been used effectively in *Lymnaea* cell culture (4.5 x 10^{-5}M) (Feng *et al.*, 1997). In this study, after 18-20 hours, intact growth cones were exposed to bath-applied anisomycin for 1 hour. To ensure that anisomycin did not adversely affect outgrowth, the rate of outgrowth in CM containing anisomycin was measured for 1 hour and compared to the rate of outgrowth in CM alone. The average rate of outgrowth in CM alone was 0.702 ± 0.459μm/min (n=10) compared to 0.73 ± 0.249μm/min (n=9) in anisomycin. No statistically significant difference was found ($p>0.05$). Next, growth cones were exposed to a gradient of *all-trans* retinoic acid (10^{-5}M) in the presence of anisomycin. These growth cones failed to turn towards the gradient, showing an average turning angle of 6.8° ± 5.8° (n=11) (Figure 11Ai-iv).

Experiments with isolated neurites were also performed. Following the 1 hour incubation in anisomycin, neurites were transected from the cell body and given 15 minutes to recover from injury. Following this period, a gradient of *all-trans* retinoic acid (10^{-5}M) was applied to the isolated growth cones. Isolated growth cones in the

presence of bath-applied anisomycin also failed to turn towards *all-trans* retinoic acid, showing an average turning angle of 1.2° ± 12.0° (n=11) (Figure 12Ai-iv). Histograms showing maximum turning angles for both intact and isolated neurites in the presence of anisomycin are shown in Figure 11B and 12B, respectively. A significant difference was found between the intact growth cone turning angle towards *all-trans* retinoic acid in CM compared to the intact growth cone turning angle to *all-trans* retinoic acid when in the presence of bath-applied anisomycin ($p<0.05$). Statistical significance was also found when the same comparison was made between the isolated growth cones in CM and the isolated growth cones in anisomycin ($p<0.05$).

These data implicate *de novo* protein synthesis in the *all-trans* retinoic acid-induced chemoattraction, and are consistent with a requirement for local protein synthesis (*i.e.*, *de novo* protein synthesis occurring within the axon/growth cone) as has been suggested for a number of other guidance cues (Campbell and Holt, 2001; Piper et al., 2006; Yao *et al.*, 2006). Figure 13 summarizes these data.

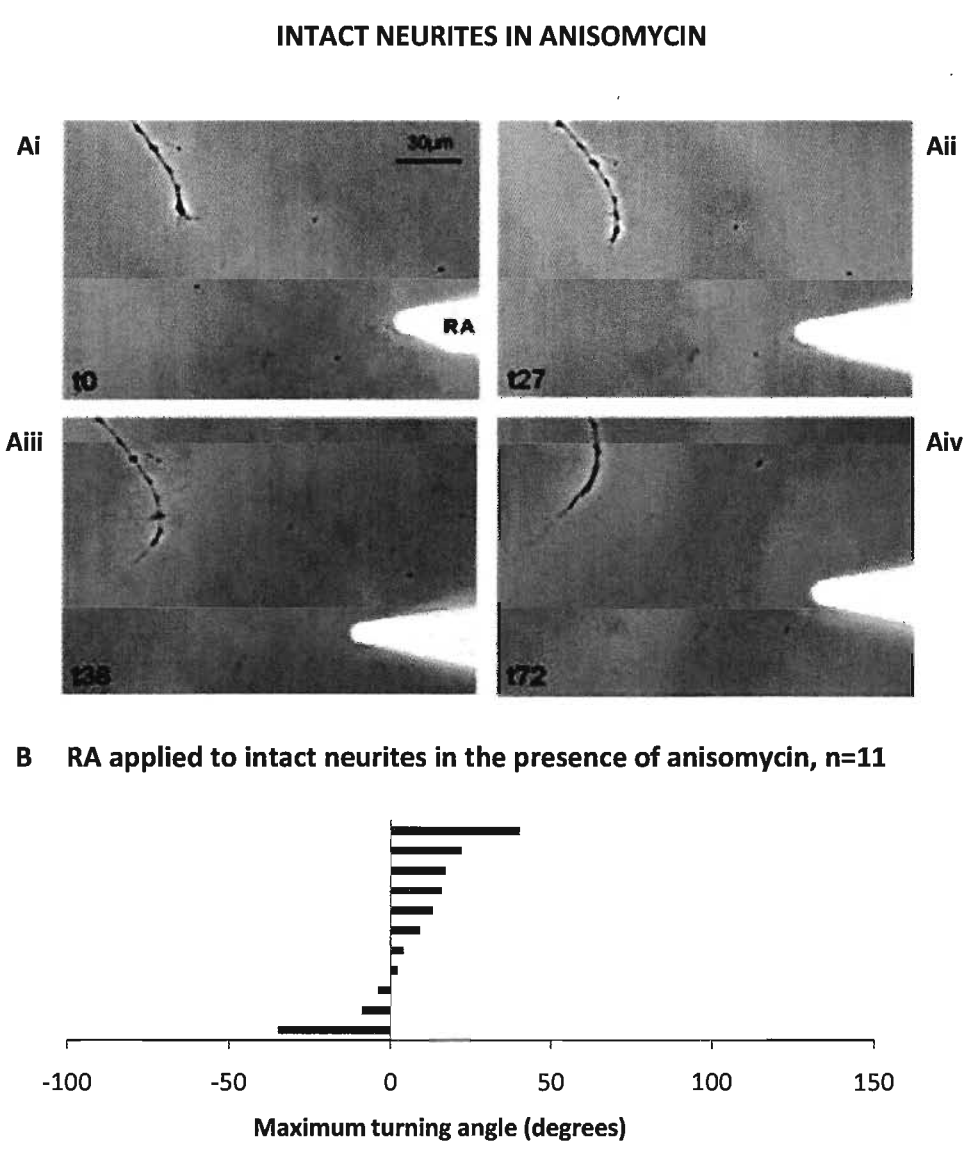


Figure 11. Anisomycin prevents intact growth cones from turning towards a gradient of *all-trans* retinoic acid. **Ai-iv.** Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid in the presence of anisomycin (4.5×10^{-5} M). Times (t) are given in minutes. **B.** Histogram shows the maximal turning angle in response to *all-trans* retinoic acid for each growth cone in the presence of anisomycin. Each bar represents one growth cone. Positive turns are towards the pipette, negative turns away from it.

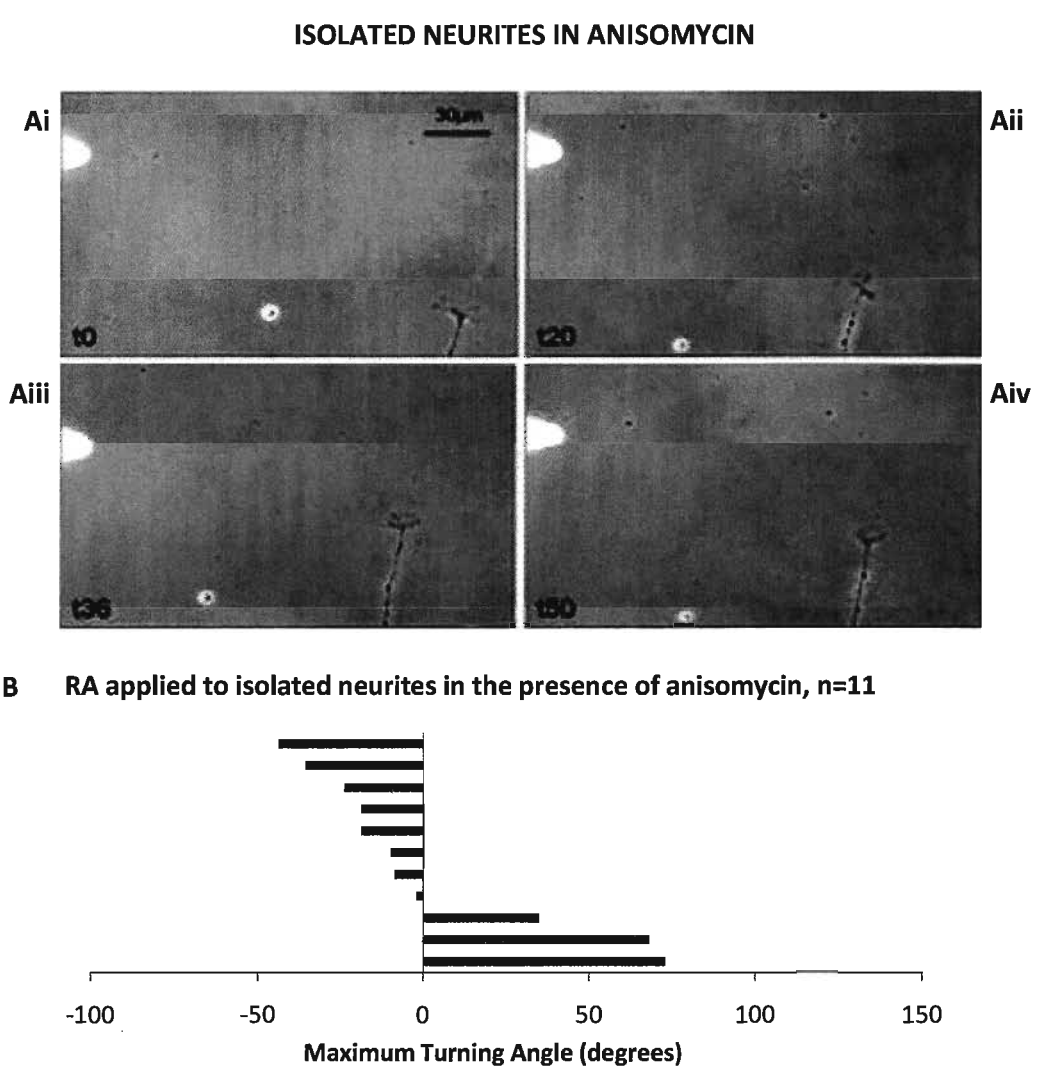


Figure 12. Isolated neurites fail to turn towards a gradient of *all-trans* retinoic acid when in the presence of anisomycin. Ai-iv. Representative micrographs showing an isolated growth cone that was not attracted by a gradient of *all-trans* retinoic acid when in the presence of anisomycin. Times (t) are given in minutes. **B.** Histogram depicting maximum turning angles for each isolated growth cone in response to *all-trans* retinoic acid in the presence of anisomycin. Each bar represents one growth cone. Positive turns are towards the pipette, negative turns away from it.

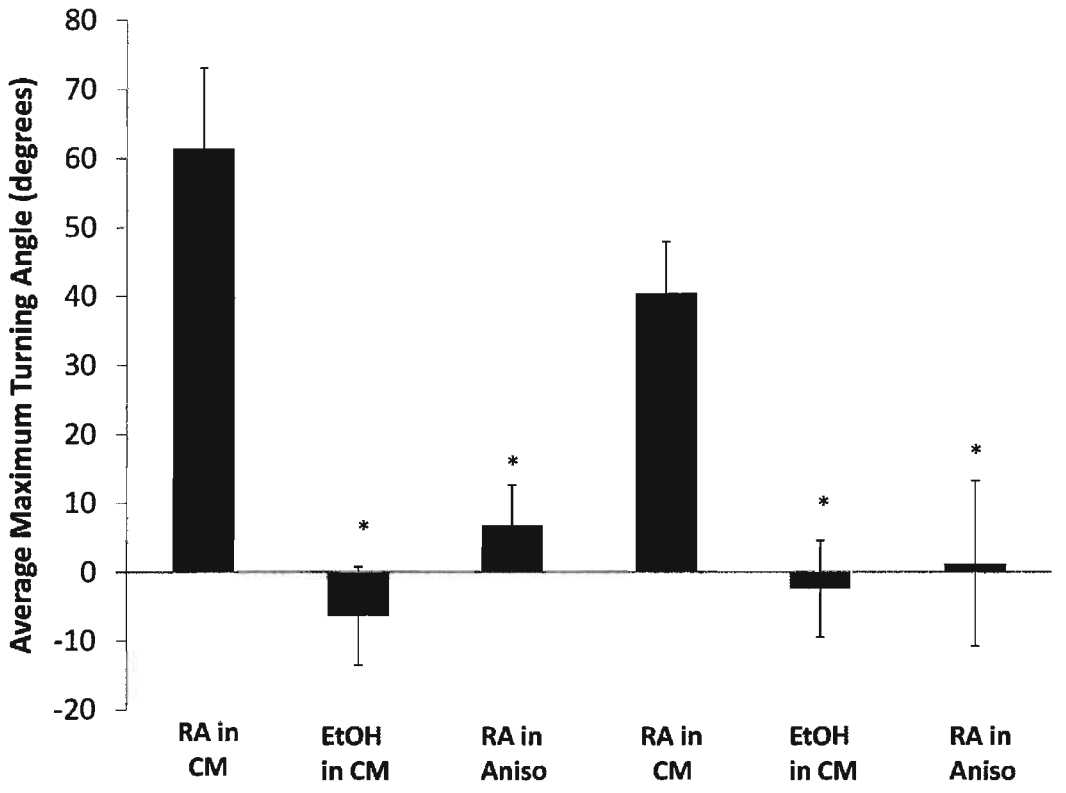
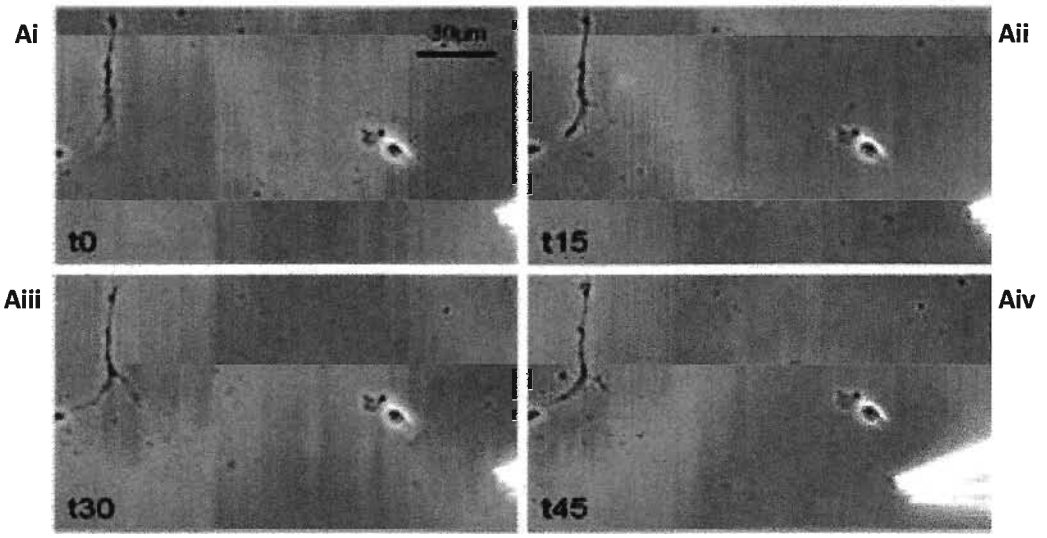


Figure 13. Summary graph showing the average maximal turning angles of growth cones in different conditions. Both the intact and isolated growth cone data are shown. Statistical comparisons are made to the *all-trans* retinoic acid application in CM alone for both intact and isolated groups. Error bars represent standard error of the mean (S.E.M.). *, $p<0.05$

3.04 Calcium influx is required for turning towards a gradient of *all-trans* retinoic acid.

Calcium influx is known to play a significant role in growth cone turning responses (Gomez and Zheng, 2006). There was also evidence for calcium signaling in a non-genomic retinoic acid signaling pathway involving neurotransmitter release at the *Xenopus* NMJ (Liou *et al.*, 2005). As there are no effective specific calcium channel blockers known to be functional in *Lymnaea*, cadmium (10^{-5}M, final bath concentration), which has previously been shown to be effective in this organism, was used (Feng *et al.*, 2000; Dmetrichuk *et al.*, 2008). Following 18-20 hours in culture, cadmium was bath-applied to neurons for 1 hour. First it was determined that incubation with cadmium had no effect on the rate of outgrowth (0.682 ± 0.369μm/min; n=10) compared to the rate of outgrowth in CM alone ($p>0.05$). Next, a gradient of *all-trans* retinoic acid was applied to growth cones in the presence of cadmium, resulting in the disruption of the normal turning response. An average turning angle of 14° ± 11.8° was found among this group (n = 15) (Figure 14Ai-iv). A histogram showing the maximum turning angles for each neurite in the presence of cadmium is shown in Figure 14B. A statistical difference was found between the growth cone turning angle towards *all-trans* retinoic acid in CM compared to the growth cone turning angle to *all-trans* retinoic acid in the presence of cadmium ($p<0.05$) (Figure 17). These data indicate that calcium influx is involved in the turning response.



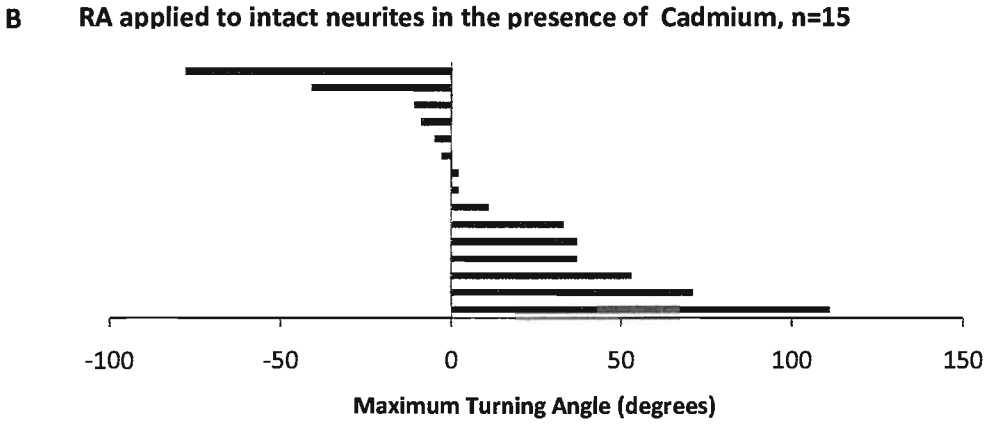


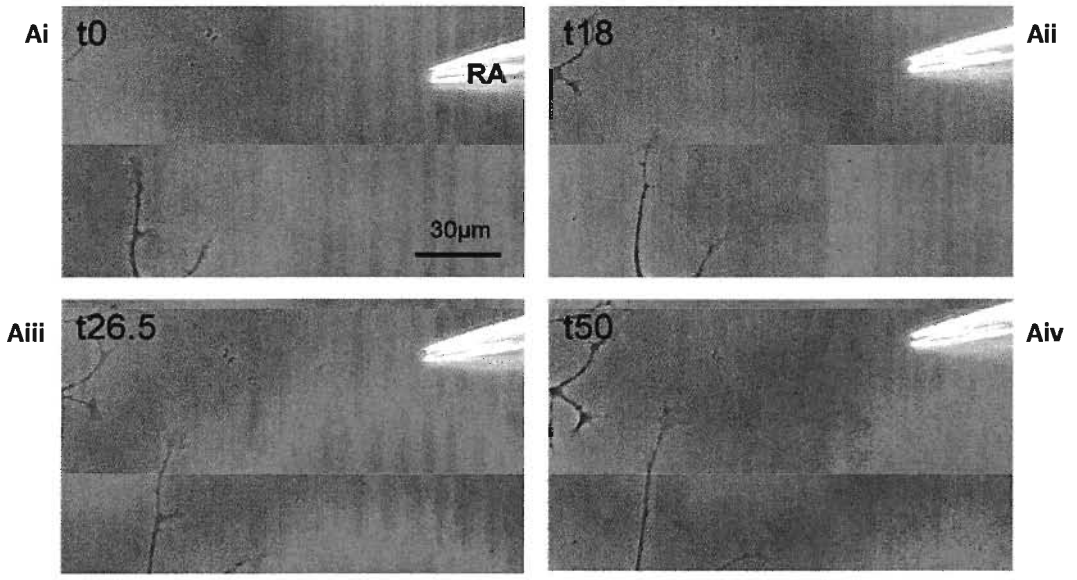
Figure 14. Cadmium significantly reduces growth cone turning towards a gradient of *all-trans* retinoic acid. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid in the presence of the cadmium. Times (t) are given in minutes. **B.** Histogram showing the maximum turning angles for growth cones exposed to a gradient of *all-trans* retinoic acid when in the presence of bath-applied cadmium. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

3.05 Protein kinase C does not appear to be required for turning towards a gradient of *all-trans* retinoic acid

Another potential target for *all-trans* retinoic acid is protein kinase C (PKC), as it has been implicated in a non-genomic retinoic acid signaling pathway and is known to possess a binding site for retinoic acid (Radominska-Pandya *et al.*, 2000; Ochoa *et al.*, 2003). Since a non-genomic pathway appeared to be at work in *Lymnaea* growth cones, it was reasonable to think that PKC may be involved. Furthermore, PKCα is known to be involved in non-genomic signaling involving calcium influx in some steroid pathways (Capiati *et al.*, 2001). To determine the potential role of PKC in the turning response, I used the PKC antagonist, Gö6976, which has previously been used effectively in *Lymnaea* cell culture (Lacchini *et al.*, 2006). First it was determined that Gö6976 did not adversely affect the rates of neurite outgrowth (0.40 ± 0.19μm/min; n=9) compared to the rate in CM alone ($p>0.05$). As DMSO was the vehicle for Gö6976, it was necessary to first test the response to *all-trans* retinoic acid in the presence of 0.01% DMSO in the bath. These control experiments showed that intact growth cones continue to turn towards a gradient of *all-trans* retinoic acid when in the presence of DMSO. However, the turning angle in DMSO is substantially, though not significantly, reduced when compared to the turning angles in CM alone, which may suggest that even low concentrations of DMSO perturb the growth cones' ability to respond to a gradient of *all-trans* retinoic acid. The average turning angle towards *all-trans* retinoic acid in the presence of DMSO was 33.6° ± 7.5° (n=11) (Figure 15).

Next, intact growth cones were tested in the presence of the PKC inhibitor. When in the presence of Gö6976, growth cones continue to turn towards a gradient of *all-trans* retinoic acid (24.1° ± 6.2° (n=10)), with an average turning angle which did not significantly differ from the response seen in the presence of the vehicle, DMSO ($p>0.05$) (Figure 16Ai-iv). However, if the growth cones exposed to *all-trans* retinoic acid in presence of Gö6976 are compared to growth cones in CM alone, a statistically significant difference is present ($p<0.05$) (*cf.* Figure 17). Therefore, the DMSO *may* be disrupting the turning response, masking the any additional effect of the PKC antagonist. Due to this possible masking effect, I was unable to unequivocally rule out the involvement of PKC in the *all-trans* retinoic acid-induced turning response.

NEURITES IN THE PRESENCE OF BATH-APPLIED DMSO



B RA appled to intact neurites in the presence of 0.01% DMSO, n=11

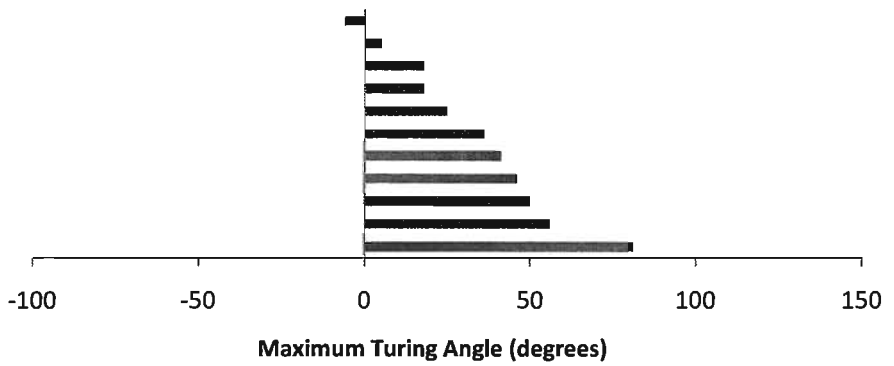


Figure 15. Intact Pedal A growth cones are attracted to a gradient of *all-trans* retinoic acid in the presence of DMSO. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid in the presence of bath-applied DMSO. Times (t) are given in minutes. **B.** Histogram showing the maximum turning angles for each growth cone towards a gradient of *all-trans* retinoic acid in the presence of DMSO. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

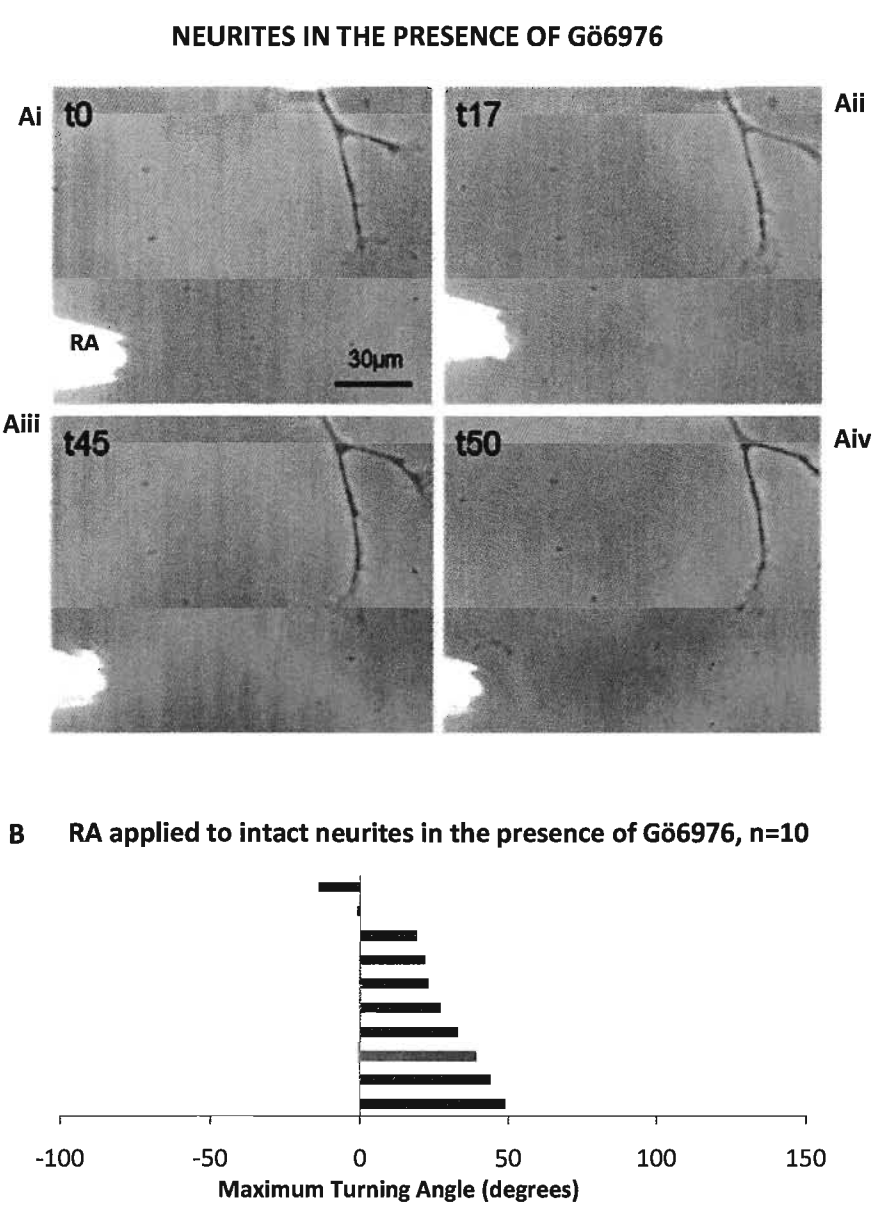


Figure 16. Intact Pedal A growth cones are attracted to a gradient of *all-trans* retinoic acid when in the presence of the PKC antagonist, Gö6976. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid when in the presence of the PKC antagonist. Times (t) are given in minutes. **B.** Histogram showing the maximum turning angles for each growth cone towards a gradient of *all-trans* retinoic acid in the presence of the PKC antagonist. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

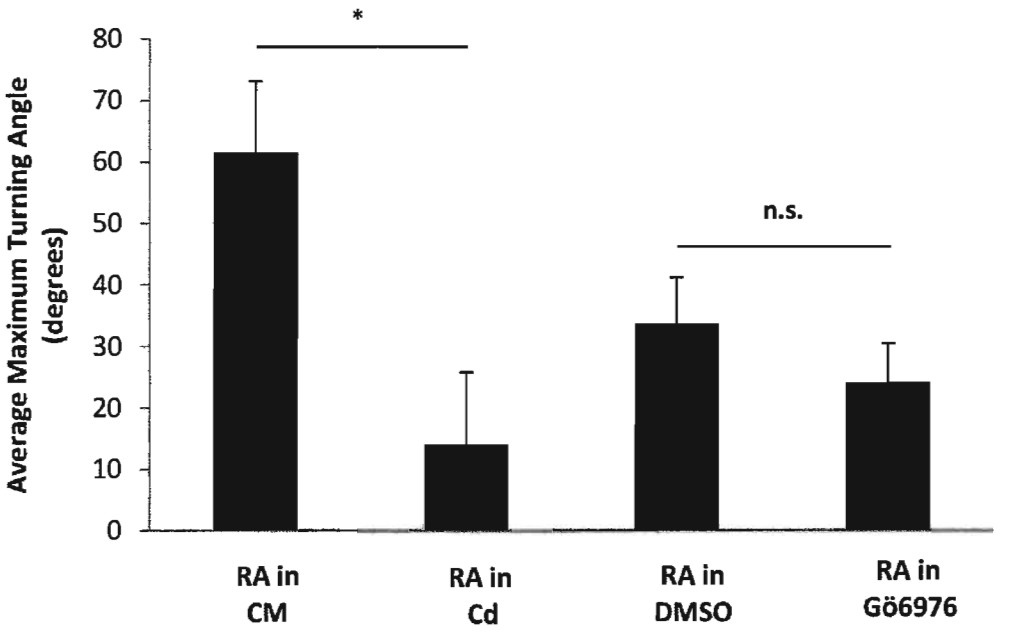


Figure 17. Summary graph showing the average maximal turning angles of growth cones in response to *all-trans* retinoic acid in CM containing cadmium, and the PKC antagonist, Gö6976. All cells were grown in CM to which inhibitors were added. Statistical comparisons are indicated by horizontal bars showing the groups compared. Error bars represent standard error of the mean (S.E.M.). n.s. = not significant, *, $p<0.05$

3.06 The role of RXR in growth cone turning

Although the RXRs are classically conceived of as nuclear receptors, and therefore would not be considered likely candidates for mediating retinoic acid-induced growth cone turning, several observations suggested that RXR may be contributing to the response in a non-genomic manner. First, after separating protein from different cellular compartments, colleague C. Carter was able to show using Western blotting that the RXR protein is present within cytoplasmic and membrane components of the *Lymnaea* CNS. Additionally, in collaboration with C. Carter, I stained for the presence of the RXR in cultured Pedal A neurons using immunostaining and found it was present in the cell body, neurites and growth cones of *Lymnaea* neurons (Figure 18).

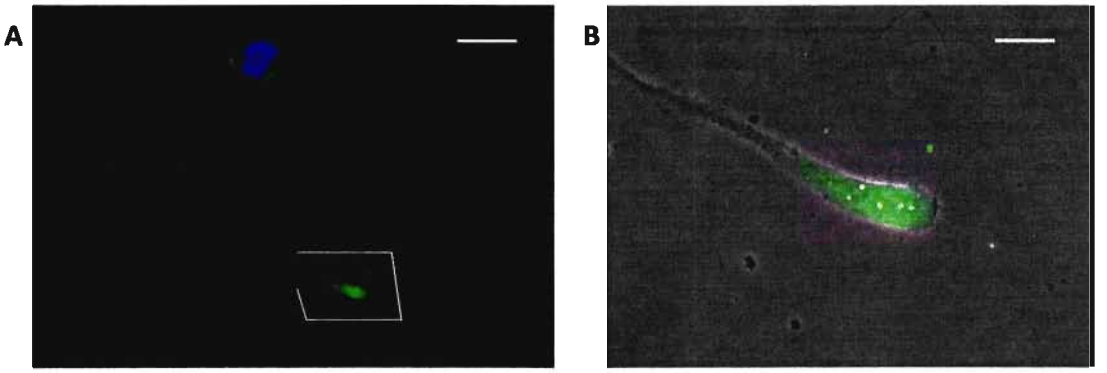
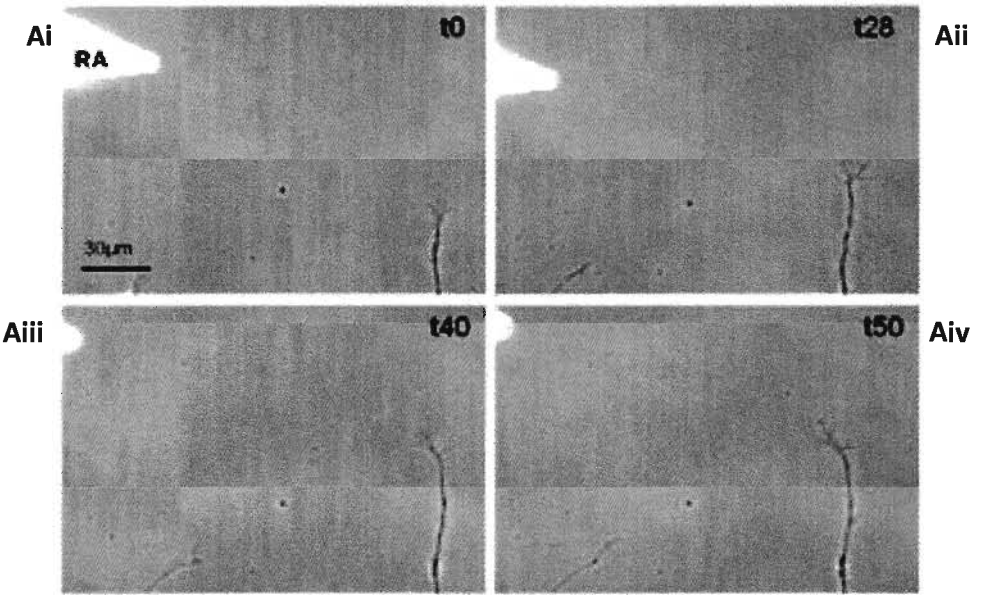


Figure 18. The RXR is present within the neurites and growth cones of *Lymnaea* pedal A neurons. A. Immunostaining shows the RXR (green) to be present in the cytoplasm, neurites and growth cones. DAPI staining outlines the nucleus (blue). Scale bar, 50µm. **B.** An enlargement of the boxed growth cone seen in A. The RXR is clearly seen within the growth cone. Scale bar, 20µm.

However, such a distribution does not necessarily imply that a non-genomic signaling pathway functions in the growth cones. Some studies, however, do support the possibility of a non-genomic mechanism involving the RARs (Liao *et al.*, 2004; Canon *et al.*, 2004; Chen and Napoli, 2008; Chen *et al.*, 2008), with some evidence for a non-genomic role for RXRs (Moraes *et al.*, 2007). Therefore, the presence of the RXR in the growth cones led me to hypothesize that it was playing a non-classical role in the retinoic acid-mediated turning response.

To test for any role the RXR may play, neurons were first exposed to the bath-applied pan-RXR antagonist PA452 (10^{-6}M), after which a gradient of *all-trans* retinoic acid was applied to growth cones. Incubation with PA452 did not adversely affect the rate of outgrowth (0.438 ± 0.477μm/min; n=10) compared to the rate in CM alone ($p>0.05$). A decreased attractive turning angle of 22° ± 3.18° (n=12) was seen in the presence of the inhibitor (Figure 19Ai-iv). Since PA452 was dissolved in DMSO, its effects were compared to the *all-trans* retinoic acid-induced turning in the presence of bath-applied DMSO; no statistically significant difference was found ($p>0.05$) (Figure 20). When the growth cones bathed in PA452 were compared to the growth cones exposed to *all-trans* retinoic acid in CM alone, a significant difference was found ($p<0.05$). This may indicate that the vehicle DMSO is masking the full effect of PA452. However, it may also indicate that the RXR is not part of the growth cone's signaling pathway. A histogram showing the maximum turning angles for each growth in the presence of PA452 is shown in Figure 19B.

INTACT NEURITES IN THE PRESENCE OF PA452



B RA applied to intact growth cones in the presence of PA452, n=12

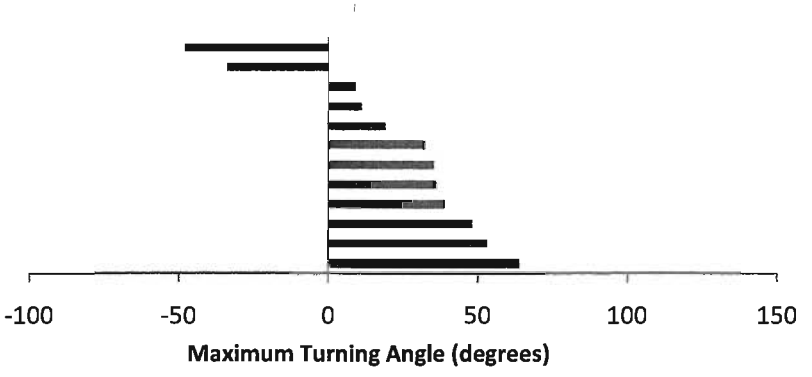


Figure 19. Intact Pedal A growth cones are attracted to a gradient of *all-trans* retinoic acid in the presence of the RXR pan-antagonist, PA452. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of *all-trans* retinoic acid in the presence of the RXR pan-antagonist. Times (t) are given in minutes. **B.** Histogram shows the maximal turning angle of growth cones in response to *all-trans* retinoic acid for each growth cone when in the presence of the bath-applied RXR antagonist. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

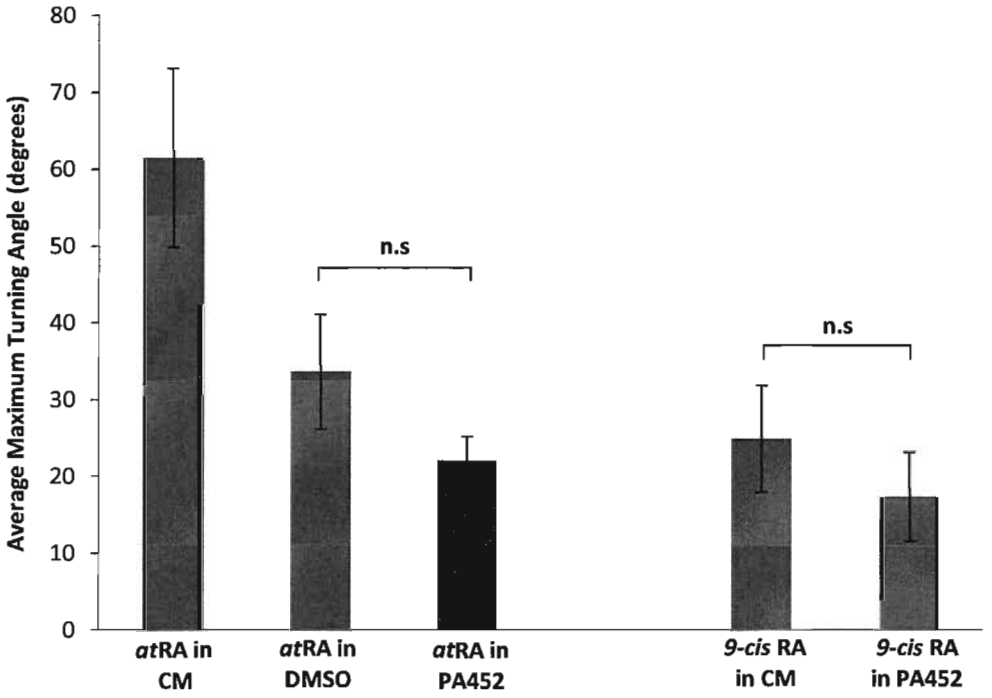


Figure 20. Summary graph showing the average maximal turning angle of intact growth cones in the presence of the RXR antagonist, PA452. All cells were grown in CM to which the inhibitors were added. Error bars represents standard error of the mean (S.E.M.), n.s., not significant ($p>0.05$) (Data labeled *atRA* in CM and *atRA* in DMSO are repeated here for comparison.)

Since *9-cis* retinoic acid is known to strongly activate the RXR (Umemiya *et al.*, 1997), and *9-cis* retinoic acid had been shown to attract the growth cones of *Lymnaea* visceral F neurons (Dmetrichuk *et al.*, 2008), I applied the RXR antagonist to cells which were exposed to a gradient of *9-cis* retinoic acid. Given evidence in the literature that vertebrate RXRs bind *9-cis* retinoic acid with higher affinity than the *all-trans* isomer, and the fact that previous studies have shown that *9-cis* retinoic acid produces growth cone turning, I hypothesized that *9-cis* retinoic acid would activate the *Lymnaea* RXR, with the RXR playing a role in the *9-cis*-mediated turning response. Like visceral F neurites, pedal A neurites were shown to be attracted towards a gradient of *9-cis* RA with an average turning angle of 24.8° ± 7°, (n=6), although the response was less pronounced compared to that seen in response to *all-trans* retinoic acid (Figure 21Ai-ii, B).

In the presence of the PA452, growth cones exposed to a gradient of *9-cis* retinoic acid continued to turn towards the gradient, with an average turning angle of 17.3° ± 5.8° (n=11) (Figure 21C). The RXR inhibitor had no statistically significant effect on the ability of growth cones to respond chemotropically to a gradient of *9-cis* retinoic acid ($p>0.05$) (Figure 20). DMSO controls were not repeated for growth cones exposed to *9-cis* RA. It should be noted that there is no evidence that the antagonist PA452 is functional with the *Lymnaea* RXR, so while these data suggested that the RXR may not be involved, an alternative approach was next used to clarify the issue.

9-*CIS* RETINOIC ACID APPLIED TO INTACT NEURITES

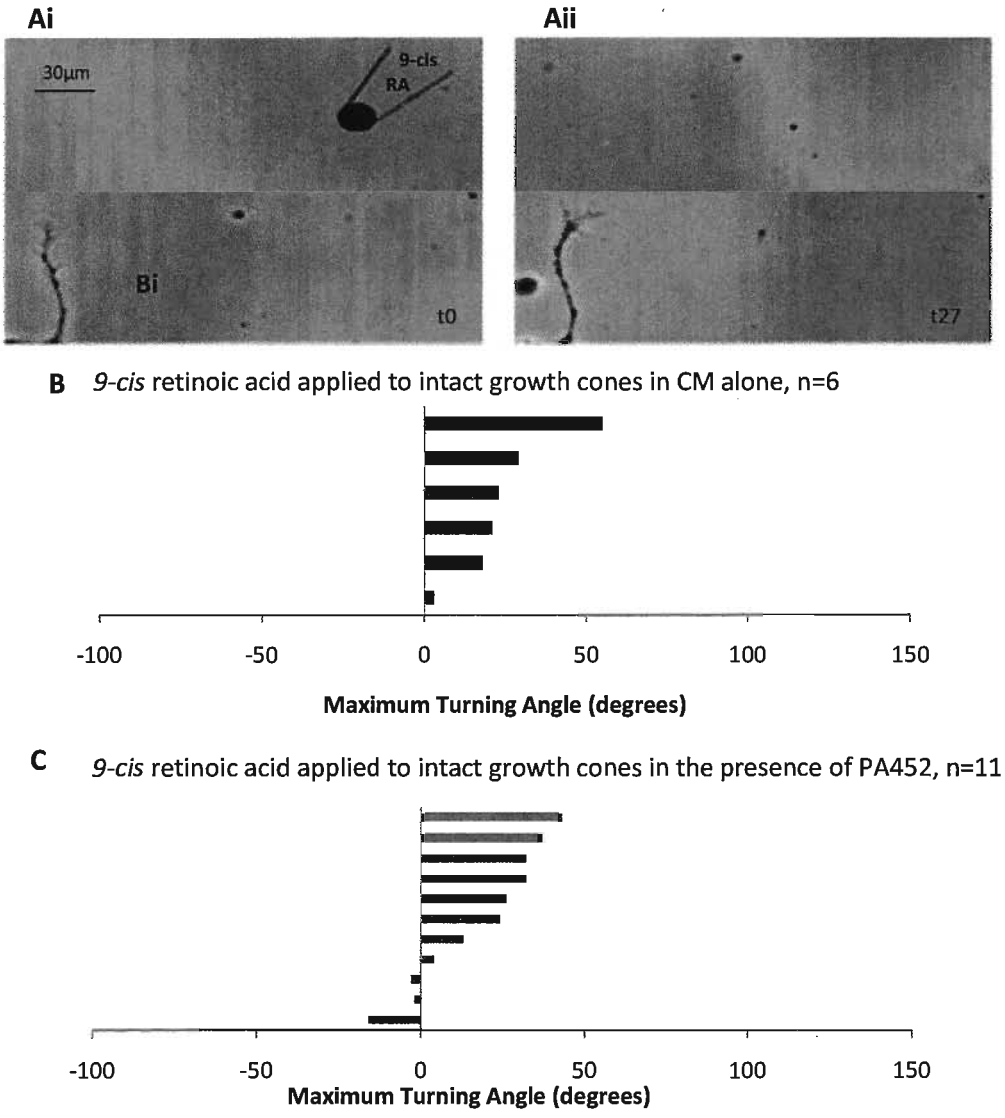
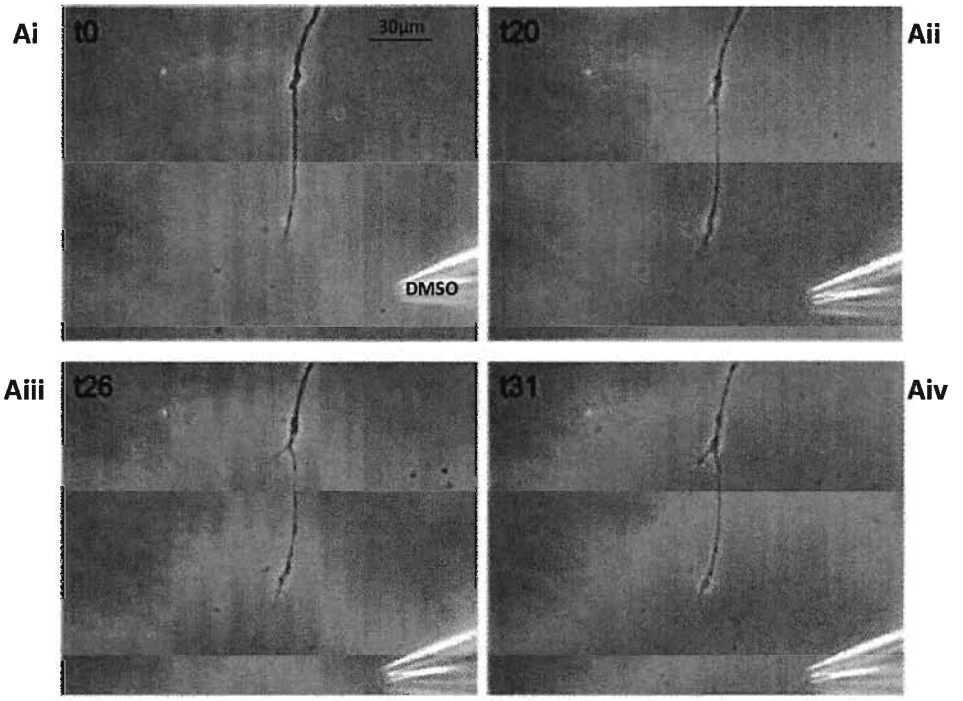


Figure 21. Intact Pedal A growth cones are attracted to a gradient of *9-cis* retinoic acid in the presence of the RXR pan-antagonist, PA452. Ai-ii. Representative micrographs showing that pedal A growth cones are attracted to a gradient of *9-cis* RA. Times (t) are given in minutes. **B.** Histogram showing the maximum turning angle of growth cones in response to *9-cis* RA. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette. **C.** Histogram showing the maximum turning angle of growth cones in response of *9-cis* RA when in the presence of bath applied PA452. Each bar represents one growth cone.

In order to further clarify the role, if any, of the RXR, I used an RXR pan-agonist in an attempt to reproduce, or mimic, a response like that seen when the growth cone encounters a gradient of *all-trans* retinoic acid. The agonist, PA024, was also dissolved in DMSO, so it was first necessary to determine that pipette application of DMSO alone did not induce growth cone turning. Growth cones were found to turn away from the DMSO gradient, exhibiting an average repulsive turning angle of -16.9° ± 8.3° (n=7) (Figure 22). Next, I tested the response to the RXR pan-agonist, PA024. When a gradient of PA024 (10^{-5}M) was applied to intact growth cones, a positive turning response was observed with an average turning angle of 30.6° ± 4° (n=14) (Figure 23Ai-iv). Comparison of the intact growth cones exposed to the gradient of DMSO and the growth cones exposed to the gradient of PA024 revealed a significant difference between these two conditions ($p<0.05$) (Figure 25). Furthermore, the growth cone response toward the RXR agonist was not significantly different from the turning angle towards either *9-cis* or *all-trans* retinoic acid, suggesting that the agonist mimicked the response to retinoic acid ($p>0.05$).

Even though the presence of the RXR in the growth cones had been demonstrated, and I had shown that the RXR agonist induces the turning of intact growth cones, these data did not rule out a genomic mechanism. Thus, my next aim was to determine whether the RXR was involved locally, within the growth cone. To this end, I determined whether the RXR agonist, PA024, could also induce growth cone turning in isolated neurites. Following the isolation of neurites, the isolated growth cones were

DMSO APPLIED TO INTACT NEURITES



B 0.1% DMSO in the pipette applied to intact growth cones, n=7

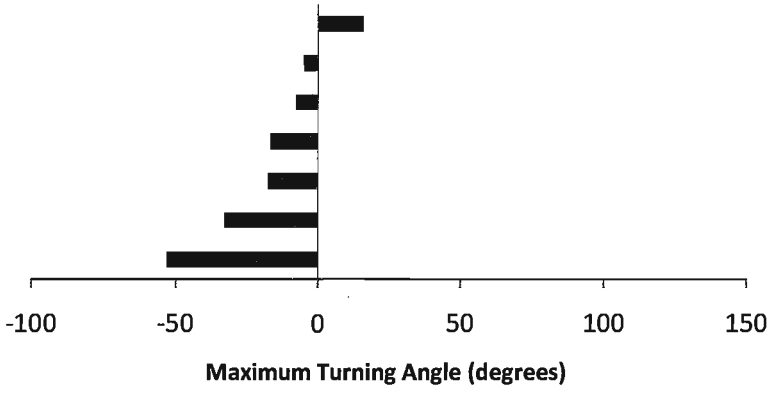
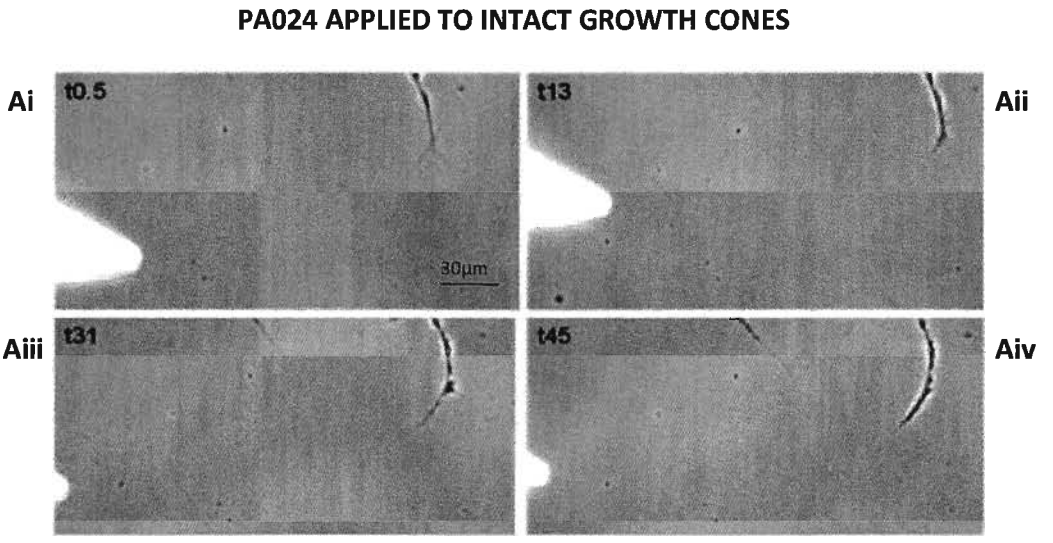


Figure 22. Pipette application of 0.1% DMSO in DM does not induce attractive turning in pedal A growth cones. Ai-Aiv. Representative micrographs showing that the vehicle DMSO in the pipette does not result in chemoattractive responses in pedal A growth cones. Time (t) is given in minutes. **B.** Histogram showing the maximum turning angle for growth cones exposed to a gradient of DMSO. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette



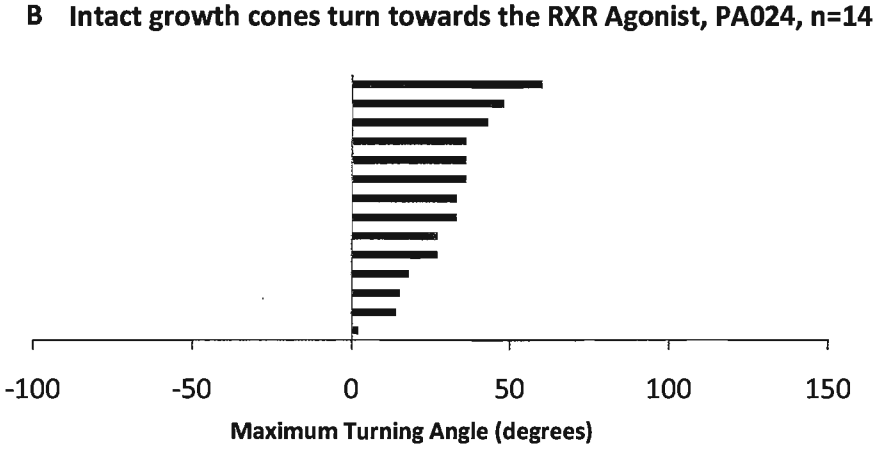


Figure 23. Intact Pedal A growth cones are attracted to a gradient of the RXR pan-agonist, PA024. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of the RXR pan-agonist, PA024. Times (t) are given in minutes. **B.** Histogram showing the maximum turning angle of growth cones in response to the RXR pan-agonist. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

exposed to a gradient of the RXR agonist, PA024. In response to the gradient of PA024, the isolated growth cones turned towards the agonist with an average angle of 24° ± 13.8° (n=11) (Figure 24Ai-iv). Comparison with the growth cones exposed to a gradient of DMSO showed a statistically significant difference ($p<0.05$) (Figure 25). However, comparison to the isolated growth cones exposed to a gradient of *all-trans* retinoic acid in CM alone found no difference ($p>0.05$), again suggesting a mimicking of the response to retinoic acid. Histograms showing the maximum turning angles in response to PA024 for both intact and isolated groups are seen in Figures 23B and 24B, respectively. Figure 25 summarizes all the data obtained with the RXR agonist. These data strongly suggest that the RXR plays a non-genomic role in the turning response of *all-trans* retinoic acid.

In summary, the data obtained in these studies show that isolated growth cones maintain their ability to turn towards a gradient of *all-trans* retinoic acid, indicating a non-genomic mechanism. Furthermore, I have shown for the first time that *all-trans* retinoic acid-induced growth cone turning requires protein synthesis, calcium influx, and likely involves a novel, non-genomic action of the RXR.

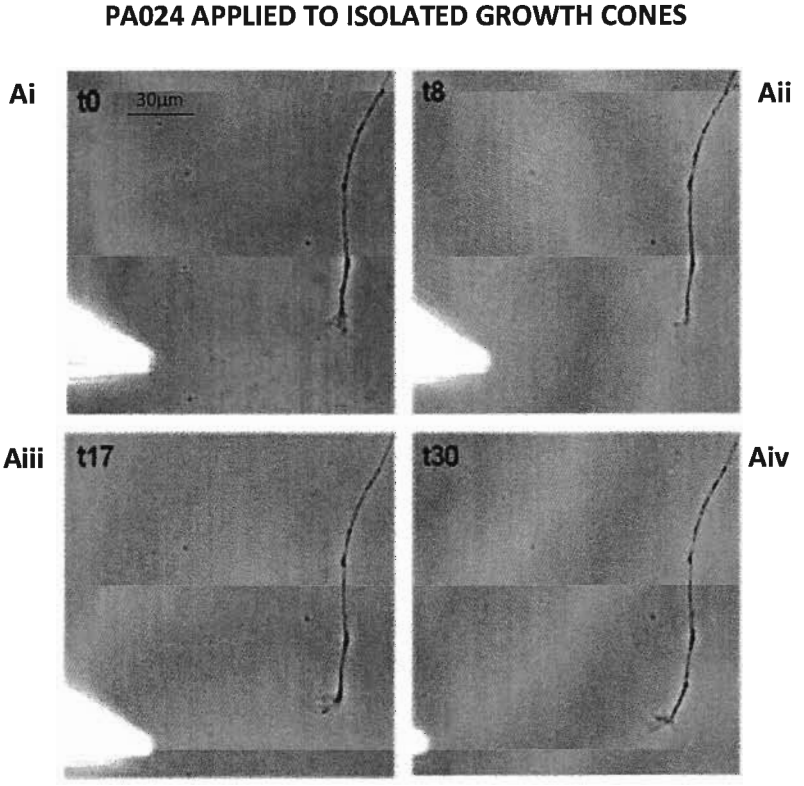


Figure 24. Isolated Pedal A growth cones are attracted to a gradient of the RXR pan-agonist, PA024. Ai-iv. Representative micrographs show the turning course of a growth cone during exposure to a gradient of the RXR pan-agonist, PA024. Times (t) are given in minutes. **B.** Histogram shows the maximum turning angle of isolated growth cones in response to the RXR pan-agonist, PA024. Each bar represents one growth cone. Positive values indicate turning towards the pipette, negative values away from the pipette.

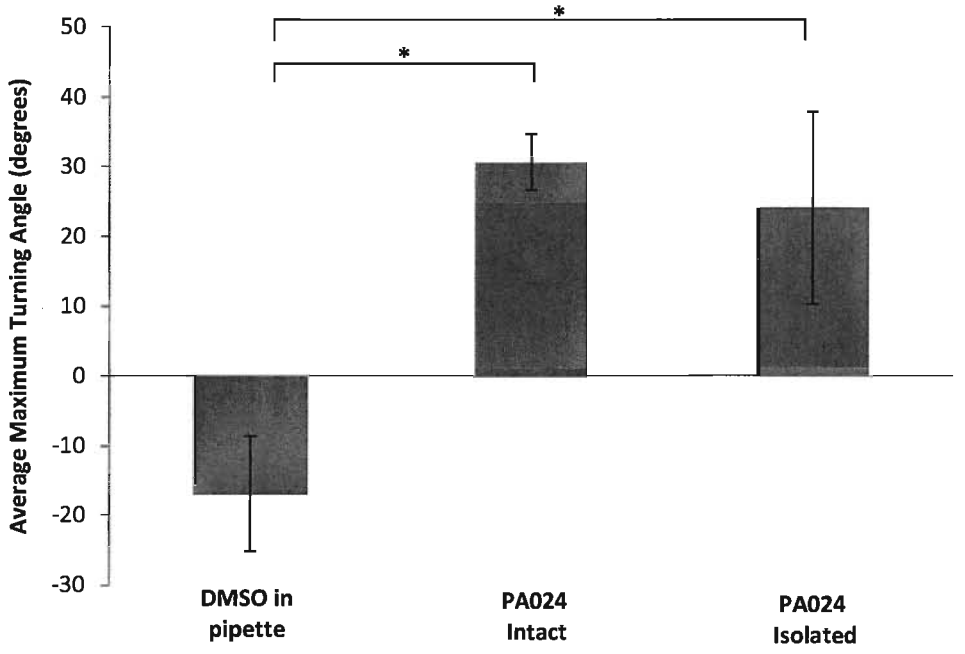


Figure 25. Summary graph showing the average maximal turning angles of growth cones in response to the RXR agonist, PA024. Statistical comparisons are made to the growth cones exposed to vehicle DMSO in the pipette. Error bars represent standard error of the mean (S.E.M.), *, $p<0.05$.

Chapter 4: Discussion

The results of my research have demonstrated that the attractive response of pedal A growth cones towards a gradient of *all-trans* retinoic acid involves a local mechanism which operates within the growth cone itself, independently of the cell body. Furthermore, this mechanism appears to involve local protein synthesis as well as calcium influx. Of special significance is the local involvement of the RXR in the turning response as its action is non-genomic, suggesting a novel role for this receptor in growth cone guidance.

4.01 *All-trans* and *9-cis* retinoic acid attract pedal A growth cones

While previous research by Dmetrichuk *et al.* (2006, 2008) had shown that visceral F neurons were attracted to gradients of both *all-trans* and *9-cis* retinoic acid, I have shown that the pedal A motoneurons are also attracted to both isomers of retinoic acid. When comparing the average turning angles for the *all-trans* experiments, no significant difference was detected ($p>0.05$), which suggests the operation of the same signaling pathway in both types of cells. Interestingly, Dmetrichuk *et al.* (2008) found no difference between the average turning angles between the VF cells exposed to *all-trans* and *9-cis* retinoic acid. However, in the pedal A neurons, the turning response to *9-cis* retinoic acid was weaker than the response to *all-trans* retinoic acid, and was weaker than the response of VF neurons to *9-cis* retinoic acid. In fact, a statistical difference was found between the turning angle towards *9-cis* retinoic acid in the VF and pedal A neurons ($p<0.05$), although both cell types clearly respond to a gradient of *9-cis* retinoic acid. It is not clear at this time why this is the case. It is possible that there is an

intrinsic difference in the sensitivity of these two cell types to *9-cis* retinoic acid. Differences in technique (*e.g.*, pressure of application, distance between pipette and growth cone, etc.) between the present studies and those of Dmetrichuk *et al.* (2008) may also account for the different results. However, this seems less likely since our findings with *all-trans* retinoic acid were very similar in the two cell types.

4.02 Isolated growth cones retain the ability to turn towards a gradient of *all-trans* retinoic acid

The complete mechanical separation of neurites from the cell body prevented any communication between the growth cone(s) and the cell body. Previous imaging studies have shown that growth cones actively send vesicles to the cell body, as well as receiving vesicles from the cell body (Denburg *et al.*, 2005). The growth cone can also rapidly communicate with the cell body through the generation of calcium waves which begin in the growth cone and travel to the cell body (Guan *et al.*, 2007). The mechanical isolation of neurites ensures that these types of communication, as well as sustained transport of proteins and mRNA molecules from the cell body to the growth cone, is permanently blocked. Therefore, the finding that isolated neurites retained the capacity to turn towards a gradient of *all-trans* retinoic acid strongly implied that a local mechanism, not requiring 'support' from the cell body, was in operation. A comparison between the average turning angle in response to *all-trans* retinoic acid in intact and isolated growth cones revealed no significant difference ($p>0.05$), confirming that isolation did not appear to adversely affect the ability of the growth cones to turn towards the gradient of *all-trans* retinoic acid.

4.03 The growth cone's response to a gradient of *all-trans* retinoic acid requires protein synthesis

Previous experiments have shown that isolated growth cones retain the ability to respond to guidance cues (Harris *et al.*, 1987; Campbell and Holt, 2001), while other studies have shown that these isolated processes initiate local protein synthesis in response to certain guidance cues (Campbell and Holt, 2001; Brunet *et al.*, 2005). However, not all guidance cues stimulate the initiation of local protein synthesis (Roche *et al.*, 2008). The reason that some cues lead to the initiation of protein synthesis (*e.g.*, Xenopus retinal ganglion cell growth cones responding to netrin-1) while others do not (*e.g.*, Ephrin-Eph signaling) is not well understood (Yoon *et al.*, 2009). Ansiomycin, previously used effectively in *Lymnaea* cell culture (Feng *et al.*, 1997), was used to show that intact pedal A growth cones require the initiation of *de novo* protein synthesis to turn towards a gradient of *all-trans* retinoic acid. Therefore, retinoic acid appears to be a guidance factor that stimulates the initiation of protein synthesis.

To determine if the requirement for protein synthesis was occurring locally within the growth cone/axon, neurites were again isolated from the cell body. As in the case of the intact neurites, isolated neurites were unable to turn towards a gradient of *all-trans* retinoic acid when in the presence of the bath-applied translation blocker, anisomycin. This finding is consistent with a role for local protein synthesis.

Intact cells were cultured for 1 hour in anisomycin before isolation of neurites, after which they were given a 15 minute recovery period, before beginning the experiment. One issue this raises is whether any proteins involved in growth cone turning had been

synthesized previously in the cell body were being shipped to the growth cone *via* axonal transport during the 1 hour exposure to anisomycin. Were this the case, the requirement for protein synthesis would not necessarily be local, despite the experiments being done in isolated neurites. Given that rates of fast axonal transport can be as high as 250μm/min, if proteins were being synthesized in the cell body, they could arrive in the growth cones within minutes (*cf.* slow axonal transport rates are approximately 15μm/min)(Vogelaar *et al.*, 2009). As Pedal A neurons in CM produced neurites averaging 673μm in length, a protein could be shipped *via* fast axonal transport from the cell body to the growth cone in less than 3 minutes. This seems unlikely since the cells had not previously been exposed to an exogenous gradient of *all-trans* retinoic acid; however, the presence of *all-trans* retinoic acid in the CM *may* stimulate the neurons to synthesize proteins needed for responding to retinoic acid. Therefore, this experimental protocol could not totally eliminate the possibility that proteins necessary for turning are synthesized in the cell body and shipped to the growth cone *via* fast axonal transport.

While the experimental protocol does not allow us to say with absolute certainty that *local* protein synthesis is occurring, there are reasons to favor this interpretation:

1) It is well established that a significant complement of mRNAs are localized in axons and growth cones. While it is true that much of the work done on axonal localization of mRNA has been done in vertebrate organisms, the earliest evidence for this localization of transcripts came from studies in invertebrates, notably squid and snails, where rRNA,

tRNA, initiation and elongation factor mRNA as well as actin, tubulin and neurofilament mRNA were detected (reviewed by Yoon *et al.*, 2009, see also Koenig, 2009). The recent demonstration of active endoplasmic reticulum and Golgi bodies within axons supports the idea that these distal domains of the neuron have the capacity to actively translate localized mRNA into *de novo* proteins (Merianda *et al.*, 2009)

Table 3. Examples of Axonally Localized mRNA Transcripts

mRNA transcript	Reference
oxytocin	Jirikowski *et al.*, 1990
vasopressin	Trembleau *et al.*, 1996
β-actin	Leung *et al.*, 2006
β-tubulin	Eng *et al.*, 1999
MAP1B	Antar *et al.*, 2006
Tau	Litman *et al.*, 1993
Neurofilament	Sotelo-Silveira *et al.*, 2000
ADF/cofilin	Lee and Hollenbeck, 2003
RhoA	Wu *et al.*, 2005
CREB	Cox *et al.*, 2008
EphB2	Brittis *et al.*, 2002
κ-opioid receptor	Bi *et al.*, 2006
Olfactory marker protein	Wensley *et al.*, 1995

A recent paper also attempted to characterize the mRNA transcripts present in neonatal rat neurons, finding mRNA transcripts generally associated with the cytoskeleton (*e.g.*, cytoskeletal building blocks and signaling molecules) (Vogelaar *et al.*, 2009). It is reasonable to suppose that with localized mRNAs in axons/growth cones and the machinery to translate them, local translation is occurring under at least some circumstances. Indeed there have been a number of demonstrations of the localized

translation of mRNA transcripts in response to external cues (as well as in response to traumatic injury (Sotelo-Silveira *et al.*, 2000)).

2) A number of guidance cues (1-5 in table below) have also been shown to trigger the translation of mRNAs within a developmental or regenerative context.

Table 4. Examples of demonstrated local protein synthesis in response to environmental stimuli

Guidance Factor	mRNA translated	Reference
1) Netrin-1	β-actin	Leung *et al.*, 2006
2) Sema3A	RhoA	Wu *et al.*, 2005
3) Slit2	Cofilin	Piper *et al.*, 2006
4) BDNF	β-actin	Yao *et al.*, 2006
5) NGF	CREB	Cox *et al.*, 2008
6) KCl depolarization	κ-opioid receptor	Bi *et al.*, 2006

Additionally, Campbell and Holt (2001) showed using radio-labeled amino acids that *Xenopus* retinal ganglion cell growth cones initiated local protein synthesis in response to netrin-1 and sema3A, by showing that proteins incorporating [^{3}H]leucine began to appear in response to a gradient of guidance factor within 10 minutes. The Holt lab has also recently used Kaede to visualize protein synthesis in *Xenopus* retinal neurons. Using a reporter construct (Kaede protein) fused to the 3'-UTR of β-actin, they were able to visualize the initiation of *de novo* synthesis of Kaede protein upon stimulation with netrin-1. Kaede is a green fluorescent protein which can be irreversibly converted to red fluorescent protein upon exposure to UV radiation between 350-400nm. By measuring the resultant Kaede signal against the baseline, they were able to show local

synthesis of Kaede protein in response to netrin-1 stimulation. Such a direct visualization of local protein synthesis in response to a guidance cue leaves little doubt that growth cones do indeed possess the capacity for local synthesis (Leung and Holt, 2008).

3) The gene products that have been shown to be locally translated in response to guidance cues are also those that would require tight regulation and control. Both RhoA and cofilin are proteins involved in remodeling the cytoskeleton, in particular, promoting its dissolution and retraction (Wu et al., 2005; Piper et al., 2006).

Therefore, it appears that many of the proteins which are locally translated in response to guidance cues are those which are required to quickly remodel the growth cone, allowing it to respond to the guidance cue.

Therefore, while the results of my experiments cannot say with absolute certainty that *localized* protein synthesis is a requirement for chemoattraction to *all-trans* retinoic acid, they are certainly consistent with such a mechanism, and such a mechanism appears probable in light of what is known about local synthesis in response to guidance cues in other organisms.

4.04 Protein kinase C does not appear to play a role in *all-trans* retinoic acid-induced growth cone turning

Given the evidence that *all-trans* retinoic acid is signaling through a local mechanism which results in the initiation of *de novo* protein synthesis, I attempted to identify target molecules with which retinoic acid may interact. Several reports have suggested that non-genomic *all-trans* retinoic acid signaling may involve activation of PKC. For

example, RA-dependent activation of CREB involves signaling involving protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and ribosomal s6 kinase (RSK) in human bronchial cells (Aggarwal *et al.*, 2006). There is also evidence for the direct modulation of PKC signaling by retinoic acid, and PKC has a retinoic acid-binding site (Radominska-Pandya *et al.*, 2000; Ochoa *et al.*, 2003). PKC has also been implicated in modulation of the cytoskeleton, which is a major end target for guidance cue signaling (Keenan and Kelleher, 1998). However, use of the PKC inhibitor Gö6976 in *Lymnaea* growth cones produced ambiguous results. Gö6976 was dissolved in DMSO, requiring an additional control to be performed (0.1% DMSO in the culture bath). The turning angle of growth cones towards *all-trans* retinoic acid when DMSO was present in the bath was decreased, albeit non-significantly, compared to the turning angle seen in CM alone. DMSO is known to disrupt the integrity of cell membranes (Yu and Quinn, 1998). As growth cone guidance depends on interactions between receptors and signaling molecules at the cell membrane, the potential disruption of the membrane by DMSO could easily lead to a compromised ability to sense and respond to guidance cues. In the presence of the PKC inhibitor, the growth cone turning response was not found to be different from the response in the vehicle DMSO ($p>0.05$). However, when compared to the response of growth cones in CM alone, a significant difference was present ($p<0.05$). This *may* indicate that the DMSO vehicle, by disrupting the integrity of the growth cone, masked a possible reduction in response to the PKC antagonist. The potential involvement of PKC cannot therefore be definitively ruled out.

Given the literature evidence for its involvement in non-genomic retinoic acid signaling, I believe further experiments are warranted. Ideally, Gö6976 would be dissolved in ethanol; however, given its low solubility, an alternate approach would need to be found. Since PKCα is the specific isoform thought to bind to *all-trans* retinoic acid, I would use dsRNA on cultured *Lymnaea* cells to knock-down expression of this protein. Application of *all-trans* retinoic acid to cells without PKCα would provide more definitive insight into any potential role this signaling molecule may be playing in the growth cone turning response, although such an approach would not necessarily suggest a direct effect of *all-trans* retinoic acid on PKCα.

4.05 Calcium influx plays a role in the *all-trans* retinoic acid-induced turning response

Calcium influx, both from the extracellular fluid and intracellular stores, is known to contribute significantly to growth cone responses (Gomez and Zheng, 2006). My results were consistent with previous reports showing the importance of calcium, as blocking calcium channels with the non-specific blocker cadmium, led to a disruption of the growth cone turning response. Therefore, growth cone turning in response to *all-trans* retinoic acid is dependent upon calcium influx from the extracellular fluid. It is interesting to note that previous work has shown a role for non-genomic retinoic acid signaling in the release of neurotransmitter at the synapse, a pathway that involved modulation of calcium levels (Liou et al., 2005). However, in contrast to the results obtained in my experiments, Liou *et al.* (2005) found that cadmium did not block the effect as the calcium influx was from intracellular stores.

It is possible that there may be a link between the calcium influx data, and the PKC data, while recognizing the latter to be somewhat ambiguous. PKCα has been shown to modulate calcium influx in the signaling pathways of some steroid hormones (Capiati *et al.*, 2001). In light of the role played by calcium in the *all-trans* retinoic acid-induced growth cone turning behavior, this is further reason to reassess a potential role for PKC.

Previous use of cadmium in *Lymnaea* cell culture has shown that a disruption of outgrowth is caused by this inhibitor after approximately 72 hours. However, during the course of my experiments the exposure time was at most 6 hours, and so any potential negative effect of the inhibitor on outgrowth was unlikely to result. The outgrowth monitoring over the first 1 hour of the experiment found no difference in rate of outgrowth when the cadmium-bathed cells were compared to those in CM alone ($p>0.05$). Cadmium was used as there are no pharmacologically-selective calcium channel blockers known to function in *Lymnaea*. However, since calcium channels from this organism have been cloned (Spafford et al., 2003), a more selective study of the channels specifically involved could be carried out using, for example, RNA interference techniques.

4.06 The RXR is involved in the turning response to *all-trans* retinoic acid

The final set of experiments conducted examined the potential role for the RXR in the *all-trans* retinoic acid-induced turning response. Classically, retinoic acid has been known to act through nuclear receptors, the RARs and the RXRs, which when bound by retinoic acid and dimerized, act as nuclear transcription factors. The finding that the

turning response was transcriptionally-independent suggested that if the RXR was involved at all it was operating in a non-genomic manner (Dmetrichuk, 2007; Farrar *et al.*, 2009). The subsequent finding that isolated neurites retained their capacity to turn towards a gradient of *all-trans* retinoic acid confirmed that if the RXR was involved it must be active in a non-genomic pathway.

While there are few known examples of the RXR functioning in a non-genomic capacity (Moraes *et al.*, 2007), there are several examples of vertebrate RARs functioning non-genomically. For example, neurotransmitter release at the neuromuscular junction can be mimicked by RARβ-specific agonists (Liao *et al.*, 2004). Activation of Erk, and subsequent phosphorylation of CREB may be triggered by retinoic acid binding to cytoplasmically localized RARs (Canon *et al.*, 2004). RARα has also been shown to repress the translation of GluR1 mRNA transcripts localized to the dendrites. The binding of retinoic acid to RARα lifts this translational repression (Poon and Chen, 2008). Conversely, there is evidence that retinoic acid can signal non-genomically, independently of its receptors (Aggarwal *et al.*, 2006), suggesting that the RXR need not be involved in the turning response.

Nevertheless, immunocytochemistry had revealed the presence of the RXR in the *Lymnaea* CNS, and its expression in the cytoplasm, neurites and growth cones of neurons (Carter *et al.*, 2008). Interestingly, a similar distribution has been noted for the vertebrate RXRα in the cytoplasm, dendrites and axons of vertebrate neurons (Calderon and Kim, 2007). The RXR cloned from *Lymnaea* also exhibits high homology with the

vertebrate RXRα (80% overall homology) (Carter *et al.* GenBank assension #AY846875). The high degree of homology between the RXR of invertebrates and vertebrates is consistent with the more recent view of an early origin for retinoic acid signaling in bilaterian animals (Campo-Paysaa *et al.*, 2008). This high degree of homology also implied that the use of RXR agonists and antagonists designed against the vertebrate RXR had a high probability of functioning in *Lymnaea* neurons, although there had been no previous demonstration of their effectiveness in this species.

The demonstration of a non-genomic role for an RXR by mimicking the response to *all-trans* retinoic acid with an RXR pan-agonist is a significant contribution to the literature. By showing that the response to *all-trans* retinoic acid could also be mimicked in isolated growth cones, the case for a localized, non-genomic role for the RXR was greatly strengthened.

Work with the RXR pan-antagonist, PA452, produced ambiguous results. Growth cones exposed to *all-trans* retinoic acid in the presence of PA452 exhibited a non-significant decrease in turning angle compared to growth cones in the vehicle DMSO ($p>0.05$). However, when compared to growth cones exposed to *all-trans* retinoic acid in CM alone, a significant difference was found. This *may* be due to a masking effect on account of the DMSO vehicle, as was perhaps seen in the case of the PKC antagonist. The application of the RXR antagonist to cells exposed to a gradient of *9-cis* retinoic acid also failed to produce a statistically significant effect on the turning response. While these data could indicate that the RXR is not involved in the turning response, the

decrease, albeit non-significant decrease, in turning *may* be indicative of a role for the RXR in the response. Despite the ambiguity of the antagonist data, the RXR pan-agonist (PA024) data point strongly to a role for the RXR in the turning response.

During the course of these studies, there was no evidence that an RAR was present in *Lymnaea stagnalis*. Genomic RAR sequences had recently been found in the mollusc *Lottia gigante*, but there was no corresponding evidence in *Lymnaea*. Thus, it was interesting that the growth cones were expressing the RXR, yet responding to a gradient of *all-trans* retinoic acid since that isomer binds to the RAR with much higher affinity, at least in vertebrates. A recent report has shown that the locust RXR binds to *9-cis* and *all-trans* retinoic acid with roughly equal affinities (Nowickyi *et al.*, 2008). Although my experimental data do not address this question, it *may* be the case that *all-trans* retinoic acid binds to the RXR with high affinity in *Lymnaea* leading to a response within the growth cones. Recently, my colleague, C. Carter, has found genetic evidence for an RAR in *Lymnaea stagnalis* (Carter *et al.*, 2009). Subsequent investigations of the mechanism underlying *all-trans* retinoic acid-induced turning should now consider the possible action of an RAR in the response.

The benefit of a local mechanism operating in the growth cone is that it permits a degree of autonomy to quickly process and respond to signals impinging on the growth cone from the environment. It is not therefore entirely surprising *in principle* to find the RXR acting in a local, non-genomic manner. The signaling pathway allows for a rapid response, in a way that a pathway involving the nucleus simply cannot. The data

currently available do not allow me to suggest a mechanism by which the RXR may be acting, but based on precedent in the literature, it may be the case that the RXR is playing a role in translational repression of localized mRNAs (as was the case with the RAR in the vertebrate dendrites (Poon and Chen, 2008)), such that stimulation by retinoic acid lifts the repression, leading to local protein synthesis. In this way, the requirement for protein synthesis and involvement of the RXR *may* be linked.

A recent report by Moraes *et al.* (2007) showed that during signaling involving *9-cis* retinoic acid in platelets, the RXR interacted with the G-protein, G_q, an association important for the signaling pathway. Given the presence of a highly conserved G_q protein in the *Lymnaea* genome, evidence for its mRNA in the CNS, and evidence for the involvement of the RXR in the turning response, this would be a promising direction to pursue (Knol *et al.*, 1995). Of relevance to this hypothesis is that disruption of G_q leads to axon guidance defects in the *Drosophila* nerve cord (Ratnaparkhi *et al.*, 2002). Also of interest was the finding in mouse hippocampal cells that G_q/G11 signaling led to the activation of Rac which promoted neurite outgrowth (Nurnberg *et al.*, 2008). There is also evidence that G_q is able to modulate calcium channels (Hildebrand *et al.*, 2007; Singh *et al.*, 2007; Bannister *et al.*, 2004; Obukhov *et al.*, 1996). Thus, one may hypothesize that the RXR interacts with G_q leading to the modulation of calcium channels in the growth cone's membrane and/or activation of Rac leading to cytoskeletal remodeling.

4.07 Summary

I have shown that like visceral F neurons, pedal A neurites from *Lymnaea stagnalis* are attracted to gradients of both *all-trans* and *9-cis* retinoic acid. The response to *all-trans* retinoic acid is dependent upon protein synthesis, which is likely occurring locally within the growth cone. Furthermore, calcium influx plays a role in the signaling mechanism. Perhaps most significant is the finding that the RXR plays a non-genomic role in the turning response. This strongly implies a novel role for the RXR in molluscan nervous system development/regeneration, and may point towards a novel pathway through which retinoic acid can signal in molluscan neurons. As many guidance mechanisms are conserved between invertebrates and vertebrates (Tessier-Lavigne and Goodman, 1996), insights derived from studying this signaling pathway in molluscs *may* be relevant to regeneration in higher vertebrates, including human beings.

Chapter 5:

Appendix 1

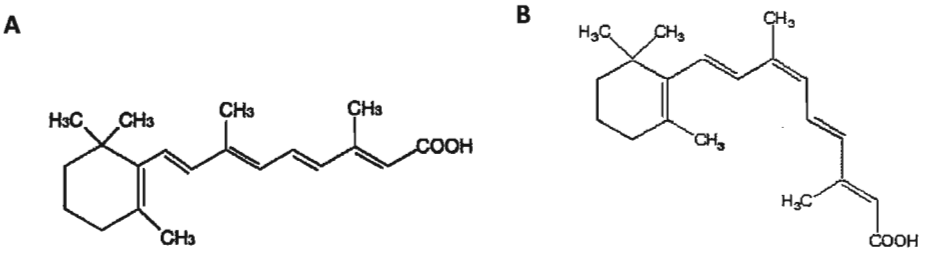


Figure 26. Two isomers of retinoic acid. A, *all-trans* retinoic acid. **B,** *9-cis* retinoic acid.

Appendix 2

Table 5. Outgrowth rates for pedal A neurons in the presence of experimentally applied pharmacological agents. All cells were grown in CM supplemented with *all-trans* retinoic acid, to which inhibitors were later added. A one-way ANOVA found no statistically significant difference across the groups. CM, conditioned media, atRA, *all-trans* retinoic acid. Data is shown as mean ± SEM.

Bath Condition	Rate of Outgrowth (μm/min)	n
CM + RA	0.702 ± 0.459	10
CM + RA + anisomycin	0.73 ± 0.249	10
CM + RA + DMSO + Gö6976	0.40 ± 0.19	9
CM + RA + DMSO + PA452	0.438 ± 0.477	10

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